Relevant MicroRNAs in Chondrosarcoma Progression

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Dedicado a la memoria de
Inesita Fernández, nuestra abuela

Contents

1 Summary

Chondrosarcoma is a malignant cartilage-forming neoplasm and the second most frequent primary bone sarcoma, representing approximately 25% of all primary bone cancers. Evidence suggests that conventional chondrosarcoma arises from pre-existing benign lesions in the bones. Despite the huge efforts done for the better understanding of this malignancy, there is still a big gap in knowledge regarding the etiology of these tumors and the genes involved in chondrosarcoma progression. Chondrosarcoma is associated with two major clinical challenges. First, these tumors are highly resistant to conventional chemo- and radiotherapeutic regimens, leaving surgical resection of tumors as the only available curative treatment. Consequently, efficient therapeutic options for patients with recurrent, metastatic or inoperable tumors are absent. Therefore, patients with advanced disease face a poor prognosis. The second challenge influencing the decision making is the current dismal accuracy for radiological and histopathological diagnostic assessment. Due to the lack of tumor-specific biomarkers, it is very arduous to discriminate chondrosarcoma tumors of different grades, and even to distinguish them from other tumors of the cartilage.

Thus, it is essential to expand the understanding of the genetic drivers involved in chondrosarcomagenesis and cancer progression. Unraveling the molecular factors contributing to chondrosarcoma pathophysiology will open up the possibility to develop targeted therapies, which may eventually improve the current clinical management and improve patients' survival rates. Additionally, novel molecules could also serve as diagnostic biomarkers that facilitate the distinction of chondrosarcoma tumor subtypes.

In order to contribute to the improvement of this defiant scenario, the research presented in this thesis focused on elucidating deregulated microRNAs (miRNAs) that are relevant for

chondrosarcoma pathogenesis. miRNAs are a family of small non-coding RNAs of approximately 20-22 nucleotides in length. They participate in a wide variety of biological processes through posttranscriptional regulation of gene expression. They play an essential role in cancer progression and uncovering their biological contribution and gene targets has great utility. Additionally, considering their origin-specificity and high stability in body fluids, we sought to also test the potential of candidate miRNAs as non-invasive biomarkers.

In summary, the results demonstrated here show that two deregulated miRNAs (miR-143/145) inversely correlate with tumor grade and play a key tumor suppressor role in chondrosarcoma *in vitro* and *in vivo*. Additionally, a combined *in silico*/sequencing approach revealed fascin-1 (FSCN1) as a direct target of miR-143/145, and its depletion phenotypically resembled miR-143/145 upregulation *in vitro*. We could demonstrate that FSCN1 overexpression is commonly found in high-grade chondrosarcoma tumors, and it contributes to the malignant phenotype of chondrosarcoma cells. Last but not least, miR-143/145 was also shown to be deregulated in plasma samples from chondrosarcoma patients. Importantly, circulating miR-145 levels can discriminate with a high accuracy between different cartilage tumor types which are nowadays hard to accurately diagnose.

2 Abbreviations

2HG	d-2-hydroxyglutarate
αKG	α-ketoglutarate
AGO	Argonaut
AKT	Protein kinase B
ATP	Adenosine triphosphate
BCL-2	B-cell lymphoma 2
CD(n#)+	Cluster of differentiation + number
COX-2	Cyclooxygenase-2
CTC	Circulating tumor cell
Dox	Doxycycline
EDTA	Ethylenediaminetetraacetic acid
EMC	Extracellular matrix components
EMT	Epithelial-mesenchymal transition
EV	Empty vector
EZH2	Enhancer of zeste 2 polycomb repressive complex 2
FAM13A	Family with sequence similarity 13 member A
FSCN1	Fascin-1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOLM1	Golgi membrane protein 1
HDAC	Histone deacetylases
HDL	High-density lipoproteins
HELLS	Helicase, lymphoid-specific
HIF-1α	Hypoxia inducible factor-1α
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule 1
IDH	Isocitrate dehydrogenase
IGF-1R	Insulin-like growth factor 1 receptor
Kb	Kilobases
miR/miRNA	microRNA

MMP	Matrix metalloproteinases
MPZL2	Myelin protein zero like 2
MRE	miRNA response element
MRI	Magnetic resonance imaging
MSI2	Musashi RNA binding protein 2
mTOR	Mammalian target of rapamycin
NAD(P)	Nicotinamide adenine dinucleotide phosphate
Nt	Nucleotides
ORF	Open reading frame
PABP	Poly(A)-binding protein
PCR	Polymerase chain reaction
PD1	Programmed cell death protein 1
PDGFR	Platelet-derived growth factor receptor
PD-L1	Programmed death-ligand 1
PI3K	Phosphoinositide 3-kinase
PTH	Parathyroid hormone
PTHRP	Parathyroid hormone-related protein
RBP	RNA-binding protein
RFP	Red fluorescent protein
RISC	RNA-induced silencing complex
RNA Pol	RNA polymerase
ROC	Receiver operating characteristic
RTKs	Receptor tyrosine kinases
SCID	Severe combined immunodeficiency
SEM	Standard error of the mean
shRNA	Short hairpin RNA
SMO	Receptor Smoothen
SOX9	Sex-determining region Y-Box 9 protein
SRGAP2	SLIT-ROBO Rho GTPase activating protein 2
TCI	Tumor cell injection
TET2	Tet methylcytosine dioxygenase 2
TF	Transcription factor

TGFB	The transforming growth factor beta
UBE2E3	Ubiquitin conjugating enzyme E2 E3
UTR	untranslated region
VEGF	Vascular endothelial growth factor
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
X-GAL	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

3 Introduction

3.1 Chondrosarcoma

3.1.1 Epidemiology and clinical characteristics

Chondrosarcoma is a malignant cartilage-forming tumor which accounts for roughly 20% of all bone tumors, making it the second-most frequent primary sarcoma of the bone, above Ewing's sarcoma and below osteosarcoma (Christopher D.M. Fletcher 2002). Its estimated overall incidence is 1 patient per 200,000 people per year. Compared to osteosarcoma and Ewing's sarcoma, which are malignancies with the highest incidences in young patients, chondrosarcoma is predominantly a malignancy of adulthood with a wide peak of incidence between 30 and 70 years old (Figure 1.A) (Dorfman and Czerniak 1995; Damron, Ward et al. 2007). Males are slightly more frequently affected than females without racial or ethnical preferences (Giuffrida, Burgueno et al. 2009; Nie, Lu et al. 2018).

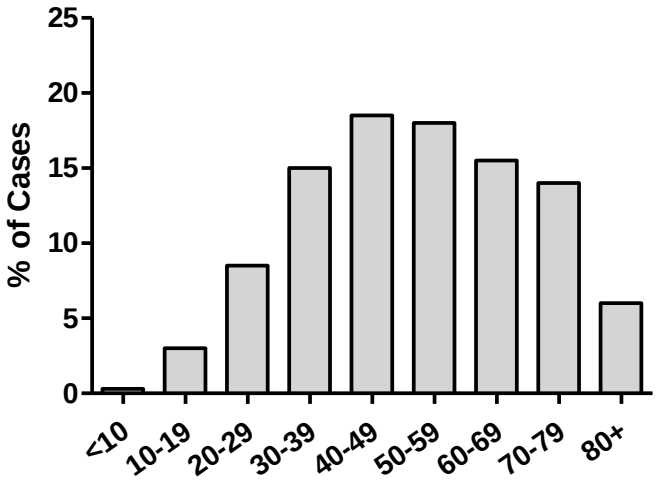
Chondrosarcoma is commonly located in the bones of the pelvis, followed by the femur, proximal humerus and ribs (Figure 1.B) and, as for most of bone tumors, long-lasting local pain and swelling are the most common symptoms (Christopher D.M. Fletcher 2002). Bone fractures can also occur in approximately 10% of patients (Björnsson, McLeod et al. 1998).

Characteristic bone lesions detected by radiography are very important for the diagnosis of chondrosarcoma. In long bones, tumors are frequently located in the metaphyseal region and they display cortical thickening of the bone, focal mineralization and sometimes bone erosion (Figure 1.C) (Soldatos, McCarthy et al. 2011).

Chondrosarcoma can be classified into three different grades (1, 2 and 3) based on histological parameters of tumors, such as cellularity, nuclear size and nuclear staining. Grade 2 and grade 3 chondrosarcomas frequently metastasize (10-50% and 53-70%, respectively) while grade 1 chondrosarcomas are very-rarely metastatic (Evans, Ayala et al.

1977; Fiorenza, Abudu et al. 2002). Patients that experience local recurrence after tumor excision most of the time develop metastasis. Metastatic lesions commonly occur in the lungs (Ozaki, Hillmann et al. 1996). Importantly, as chondrosarcoma tumors are virtually non-responsive to chemotherapy and radiotherapy, clinical management is limited only to surgery (Gelderblom, Hogendoorn et al. 2008).

A)



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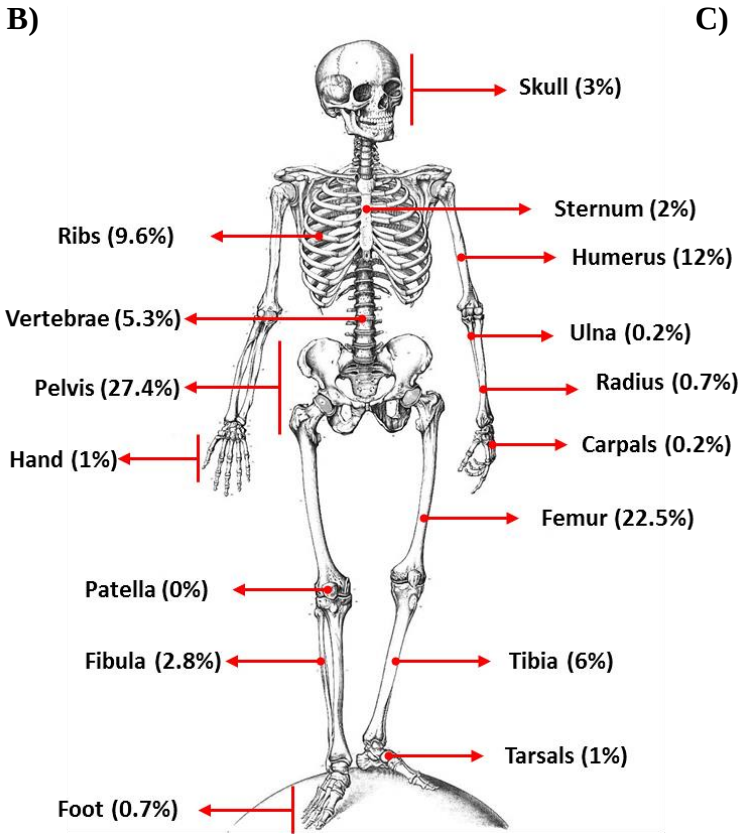

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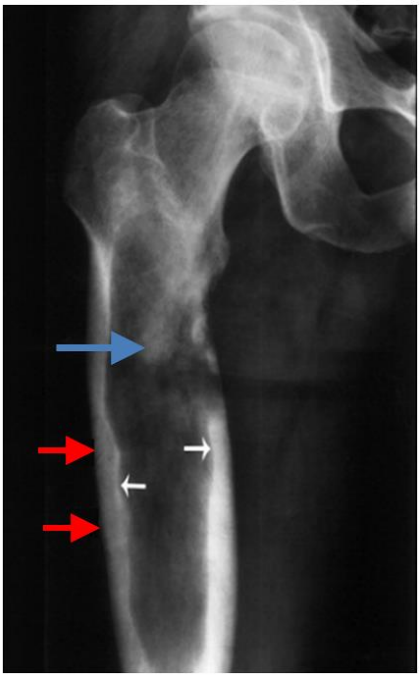


Figure 1: A) Percent distribution of chondrosarcoma patients per age. Adapted from (Damron, Ward et al. 2007). **B)** Frequency distribution of chondrosarcoma tumors across the skeleton. Figure adapted from (van Oosterwijk, Anninga et al. 2013). **C)** X-ray radiography showing a chondrosarcoma tumor presenting lytic lesion in the right femur with cortical endosteal scalloping (erosion of the inner cortex, white arrows), chondroid matrix production (blue arrow) and cortical thickening (red arrows). Adapted from (Littrell, Wenger et al. 2004)

3.1.2 Chondrogenic tumors

In order to understand how chondrosarcoma arises (or better said: how it possibly arises, as it will be discussed in section 3.1.3 "*Etiology*") it is first necessary to remark that chondrosarcomas can originate from certain preexisting benign tumors. In addition, chondrosarcomas can also give origin to other malignant tumors of the human cartilage. Thus, because of the interconnection and the radio-histological similarities between them, they are frequently addressed as a broad group of tumors. Ergo, this part of the thesis aims to summarize and give a clarified picture of the different chondroid-matrix producing neoplasms, that together with chondrosarcoma, are commonly referred as cartilaginous or chondrogenic tumors (Fletcher, Unni et al. 2002). Importantly, all chondrogenic variants, benign and malignant, are recognized by the World Health Organization (WHO) (Table 1).

One commonality among all the members of this broad group is that they are all believed to develop at the growth plate of bones. Primary chondrosarcoma is the most common cartilaginous neoplasm and the most studied one. Depending on the location of the tumors, two chondrosarcoma types can be recognized: central (also known as *conventional chondrosarcoma*) and peripheral. The first ones occur at the medullary cavity of bones, whereas the latter ones originate in the surface of bones, and although histologically indistinguishable, they have genetic differences (Bovée, van Royen et al. 2000).

Evidence supports that the majority of chondrosarcomas emerge from preexisting benign tumor entities, specifically from enchondromas and osteochondromas, given their similar

histological appearance and tumor localization. Enchondromas develop in the bone cavity and, therefore, are thought to progress towards central chondrosarcoma. Similarly, for being superficial bone tumors, osteochondromas can evolve into peripheral chondrosarcoma. Both benign tumor types have high survival rates and, typically, complete tumor removal is curative. Other benign tumors are recognized (chondroblastoma, periosteal chondroma and chondromixoid fibroma), but due to their unknown/unclear link to chondrosarcoma, they will not be discussed in the present thesis.

Several malignant chondrogenic variants also exist and, although they show some commonalities with central chondrosarcoma, they are still poorly characterized tumor types. The first one is dedifferentiated chondrosarcoma, which accounts for 10% of all chondrosarcoma types and it is characterized by a mixture of two defined neoplastic components: a cartilaginous tumor juxtaposed with a non chondrogenic sarcoma, which can be osteosarcoma, fibroma, rhabdomyosarcoma or others. Importantly, dedifferentiated chondrosarcoma may arise from preexisting conventional chondrosarcoma tumors (Bovee, Hogendoorn et al. 2010). As it will be addressed in more detail in section 3.1.4.1 (*Metastasis in chondrosarcoma*), the dedifferentiated is the most aggressive chondrosarcoma variant with a 5-year overall survival rate lower than 10%. A second rare chondrosarcoma subtype is a highly malignant and recurrent tumor named mesenchymal chondrosarcoma (3–10% of all chondrosarcomas) which is histologically seen as a mixture of normal cartilage cells together with small undifferentiated cells. Lastly, clear cell chondrosarcoma is a low grade variant comprising for 2% of all chondrosarcoma that is occurring in the epiphyseal region of long bones and it is histologically defined by the presence of clear cells mixed with cartilage (Christopher D.M. Fletcher 2002).

Tumor type	Frequency
Benign	
Enchondroma	34%
Osteochondroma	30%
Chondroblastoma	19%
Periosteal chondroma	10%
Chondromyxoid fibroma	7%
Malignant	
Conventional central chondrosarcoma	75%
Secondary peripheral chondrosarcoma	10%
Dedifferentiated chondrosarcoma	10%
Clear cell chondrosarcoma	2%
Mesenchymal chondrosarcoma	2%
Periosteal chondrosarcoma	1%

Table 1: Chondrogenic tumor types according to the WHO. Figure adapted from (Bovee, Hogendoorn et al. 2010)

3.1.2.1 Grading of chondrosarcoma tumors and its challenges

Conventional chondrosarcoma tumors can progress from poorly aggressive non-metastatic variants (Grade 1) to malignant ones (Grade 2 and 3). The assessment of the grading of chondrosarcoma is based on different histological and imaging features such as cellularity, nuclear atypia, vascularization and aberrant production of extracellular matrix components (EMC) within the tumor, with all these factors markedly increasing with tumor grade as shown in Figure 2 (Christopher D.M. Fletcher 2002). Collagens (2, 2a and 11), hyaluronan, aggrecan and heparin sulfate are possibly the best described ECM components in chondrosarcoma (Aigner, Dertinger et al. 1997; Schrage, Hameetman et al. 2009; Totoki, Yoshida et al. 2014; Hamada, Nishida et al. 2018).

Due to its complexity and lack of reliable biomarkers, grade assessment is known to be an arduous task for pathologists, leading with frequency to the erroneous categorizations of tumors, especially between Grade 1 and Grade 2 chondrosarcoma cases (Eefting, Schrage et al. 2009). The improvement of this low reliability is of outmost relevance for the field, as tumor grading is a strong prognostic factor. Additionally, because Grade 1 chondrosarcomas are treated differently than Grade 2 and 3 (as described in detail in Section 3.1.5, *Chondrosarcoma treatment*), wrong grading assessment has a direct negative impact on clinical management too. Moreover, benign chondrogenic tumors, especially enchondromas, can be extremely hard to differentiate from Grade 1 and sometimes even from Grade 2/3 chondrosarcomas (SLICED 2007), a fact that may explain the marked variation between chondrosarcoma outcomes at different clinical centers. Taken all these facts together, the discovery of sensitive biomarkers that are specific for tumor subtypes, or the development of better imaging methodologies to discriminate them, are both indispensable to improve the current dismal accuracy.

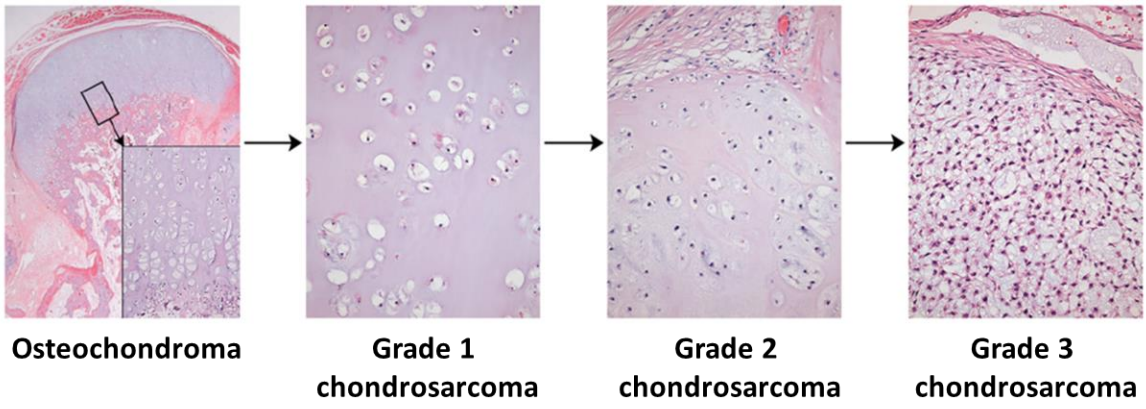


Figure 2: Changes seen in the cellularity and extracellular matrix distribution during the malignant progression of osteochondromas towards peripheral chondrosarcoma. Figure adapted from (Hameetman, Rozeman et al. 2006)

3.1.3 Etiology

Despite the numerous efforts in chondrosarcoma research, its cell origin still remains unknown. Nevertheless, a large amount of data supports that chondrosarcomas originate from benign chondrogenic neoplasms, being enchondromas and osteochondromas, which are thought to develop from the growth plate of bones. The growth plate is the region between the epiphysis and the metaphysis of long bones (Figure 3.A). The cartilage cells within the growth plate, named chondrocytes, experience a coordinated process of cell differentiation which ends up in the formation of hypertrophic chondrocytes. These enlarged hypertrophic cells are genetically programmed to secrete different components to the extracellular matrix space which are needed for new bone formation. In addition to this, hypertrophic chondrocytes engage blood vessels and osteoblasts resulting in newly synthesized bone. It is through this process of chondrocyte enlargement and osteoblast attraction that longitudinal growth of bone occurs, which is commonly referred to as *endochondral bone formation*.

From an anatomical point of view, the strongest evidence supporting that enchondromas and osteochondromas arise from chondrocytes of the growth plate are tumor location and stage of life of appearance. Both tumors are known to arise at the metaphyseal portion of the bone. As newly generated bone originates in that same region, it is likely that these benign tumors are product of an erroneous endochondral bone formation process. The second important aspect is that enchondromas and osteochondromas usually develop before adulthood (even if the detection is done during the adulthood, possibly because they are not painful), meaning, at the time when bones are still enlarging in the body and chondrocytes can still actively divide.

From a genetic perspective, an increasing amount of evidence reinforces the hypothesis which suggests that the growth plate is the site of origin of these benign entities, that

subsequently progress towards chondrosarcoma. Briefly, there are two main signaling pathways responsible for bone development during endochondral ossification: the Indian Hedgehog (IHH) and the Parathyroid hormone and Parathyroid hormone-related peptide (PTH/PTHrP) pathways. The IHH pathway is responsible for chondrocyte differentiation and said in other words, it drives the conversion of normal chondrocytes towards pre- and hypertrophic chondrocytes (Kobayashi, Chung et al. 2002). The PTHrP pathway, on the other hand, guarantees chondrocyte proliferation in the growth plate. Both pathways act in a negative feedback loop and this link suggests that their interactions regulate the right pool of proliferative and hypertrophic chondrocytes (Figure 3.B) (Kronenberg 2003). Importantly, members of both pathways have been reported to be constitutively active in benign chondrogenic tumors (Tiet, Hopyan et al. 2006). In addition to this, the deregulation of the signaling members of both pathways is more pronounced in high-grade chondrosarcomas, suggesting that IHH and PTH/PTHrP are not only required for chondrosarcomagenesis, but also for cancer progression (Bovee, van den Broek et al. 2000).

Given the importance of the IHH/PTHrP signaling axis, many targeted-therapies have been tested against protein members of these pathways, as it will be addressed in more detail in section 3.1.5 (*Chondrosarcoma treatment*).

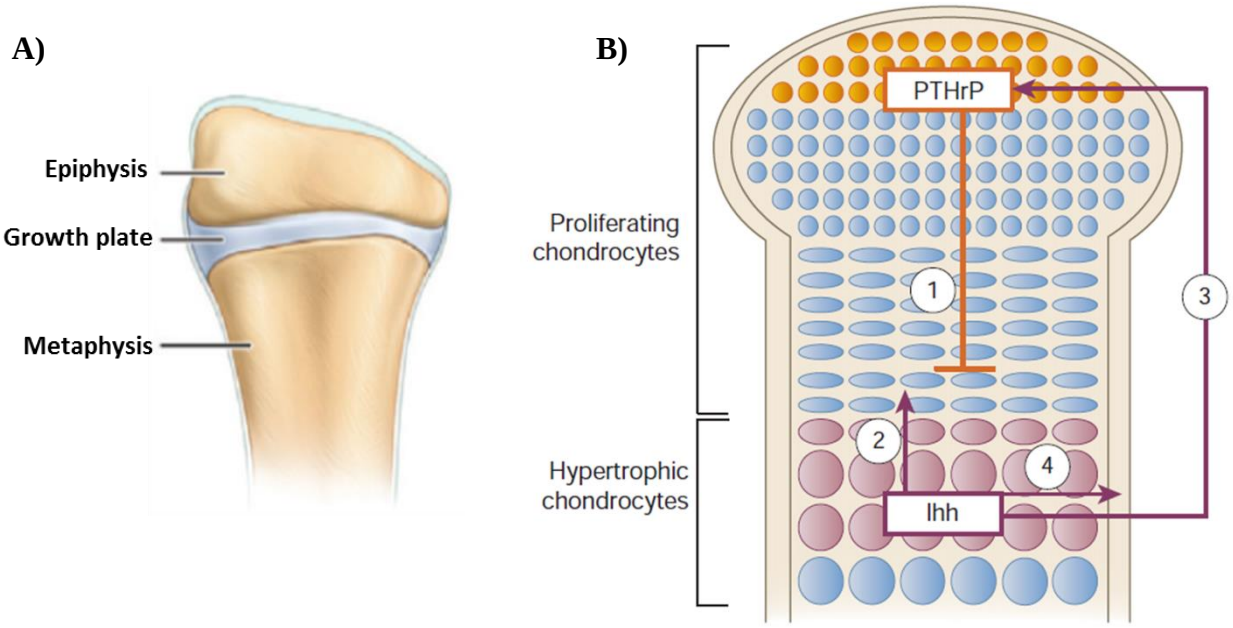


Figure 3: A) Schematic representation of the different regions at the terminal bone anatomy**.** Adapted from (Little, Klionsky et al. 2014). **B)** The IHH and PTHrP signaling pathways are major players in regulating differentiation and proliferation of chondrocytes of the growth plate. The PTHrP pathway delays chondrocyte differentiation by downregulating IHH activation (1). IHH signaling (through Gli transcription factors) drives chondrocyte differentiation causing the longitudinal growth of the bones (2). Additionally, IHH signaling directly upregulates PTHrP expression at the end of bones (3) by an unclear mechanism. IHH also stimulates the recruitment of perivascular cells and osteoblast and osteoclasts in order to produce new bone. In the case of benign chondrogenic tumors and chondrosarcoma, there is deregulation of this feedback loop, resulting in constitutive activation of IHH signaling. Figure adapted from (Kronenberg 2003)

3.1.4 Metastasis

Metastasis is the main cause of mortality in chondrosarcoma patients, and in all cancer types as a general rule (Mehlen and Puisieux 2006). Although far from being fully understood, the formation of metastasis relies on a chain of multiple coordinated events that result in the dissemination of cancer cells into distant tissues, a process commonly known as "the invasion-metastatic cascade" (Valastyan and Weinberg 2011). The very first step in this cascade consists on the initial invasion of the tumor cells into neighboring normal or neoplastic tissues. This step is followed by the migration of the cancer cells across the surrounding blood or lymphatic vasculature (intravasation). Once in the lumen of vessels, the circulating tumor cells

(CTCs) spread throughout the body as part of the normal circulating transit. To increase their survival chances CTCs are known to bind to normal circulating cells (platelets and neutrophils mainly) (Leblanc and Peyruchaud 2016; Szczerba, Castro-Giner et al. 2019). Importantly, those very few cells that manage to survive the immune attack as well as to the oxidative and mechanical stress can eventually exit the vasculature barrier and colonize distant organs (extravasation). Lastly, tumor cells that reach the parenchyma can divide and form small colonies (micrometastases) which after several subsequent cell division rounds can give formation to macroscopic metastases (macrometastases).

In cancer of epithelial origin (carcinomas), a central mechanism in the metastatic process is the epithelial-mesenchymal transition (EMT). When going through EMT, epithelial cancer cells undergo a genetic reprogramming process that results in the loss of some of its epithelial features and the subsequent gain of some mesenchymal-like phenotypic properties. These changes confer to cancer cells multiple advantageous features such as an increased invasive potential, resistance to chemo- and radiotherapeutic agents or an enhanced ability to degrade extracellular matrix components (EMC) (Hanahan and Weinberg 2011). From a genetic regulatory standpoint, EMT requires the coordination of multiple transcription factors (TFs) for the activation of numerous regulatory gene networks that results in the molecular reprogramming of the cancer cells.

In contrast, in cancer types of mesenchymal origin like chondrosarcoma, the role of EMT-related processes is very unclear. In fact, linking EMT with sarcoma might sound paradoxical, as primary tumors are mesenchymal *ab initio*. Still, some evidence suggests that specific sarcoma subtypes might experience EMT-like processes. For example, the inverse process to EMT, named mesenchymal-to-epithelial transition (MET), has been noted in sarcomas. This is characterized by an (incomplete) epithelial differentiation of the tumor cells, resulting in

lowered tumor malignancies. This is supported by the fact that expression of conventional EMT TFs, such as Snail, Slug and Twist has been reported in sarcomas. This suggests the possibility that certain sarcomas may experience a mixed state characterized by the presence of both, epithelial and mesenchymal features (Kahlert, Joseph et al. 2017; Sannino, Marchetto et al. 2017) Although an increasing amount of evidence supports that some sarcoma types may go through EMT-like processes that contribute to cancer malignancy, the field is still far from being fully understood and additional studies are required to unravel the mechanisms governing these phenomena.

3.1.4.1 Metastasis in chondrosarcoma

Metastasis is the main cause of mortality in patients with chondrosarcoma and 23% of all patients develop metastasis at some point during their disease. However, if one does not take into account all chondrosarcoma Grade 1 cases – which are commonly acknowledged as non-metastatic- the remaining combined chondrosarcoma Grade 2 and 3 metastasize on average in 50% of all patients (Evans, Ayala et al. 1977; Fiorenza, Abudu et al. 2002). Due to the direct link of grading with high risk of developing metastasis, it is key predictor of clinical behavior in chondrosarcoma. The five-year survival for chondrosarcoma Grade 1 patients is around 90%, while for chondrosarcoma Grade 2 and Grade 3 is 60% and 40% respectively (Figure 4.A) (Fiorenza, Abudu et al. 2002; Andreou, Ruppin et al. 2011). Metastases occur with a strong preference in the lungs, accounting on average approximately 80% of all metastatic cases. Other less common sites include skin, soft tissue, brain, spine and liver (Evans, Ayala et al. 1977; Ozaki, Hillmann et al. 1996).

Approximately 10% of all chondrosarcoma patients develop tumors that cannot be treated surgically, usually referred to as unresectable or non-resectable conventional chondrosarcoma.

The most common causes of unresectability are tumor location, tumor size and enhanced metastatic spread at time of diagnosis. Most unresectable tumors originate at the skull and the pelvis, making wide-margin surgeries at these sites very challenging (Bloch, Jian et al. 2010). These patients face worse survival rates than patients with resectable tumors (Figure 4.B) (van Maldegem, Gelderblom et al. 2014).

Not surprisingly, benign chondrogenic tumors (enchondromas and osteochondromas) do not metastasize. Nevertheless, as they can progress towards conventional chondrosarcoma, they may result in metastasis development if not treated on time. On the contrary, dedifferentiated chondrosarcomas are highly metastatic resulting in 5-year survival rates between 7% and 18%, making it the most lethal chondrosarcoma variant(Christopher D.M. Fletcher 2002).

Little is known about the molecular mechanisms behind chondrosarcoma metastasis in the lungs. It has been reported that upon methylation of CpG islands, chondrosarcoma cells experience an EMT-like process, characterized by the changes in the expression of different epithelial markers (Fitzgerald, Gourronc et al. 2011). A positive correlation between Twist1 levels and tumor grade was also reported in chondrosarcoma (Wu, Huang et al. 2016). Several other studies have identified various gene candidates that can be linked to the metastatic process in chondrosarcoma, but the amount of functional evidence is still insufficient. For example, the expression of several Matrix metalloproteinases (MMPs) was associated with the metastatic potential of chondrosarcoma tumors (Hou, Hsiao et al. 2009; Malcherczyk, Heyse et al. 2018). Additionally, upregulation of the intercellular adhesion molecule-1 (ICAM-1) – a gene linked with metastasis in some carcinomas- was found to increase the migratory capacity of chondrosarcoma cells (Fong, Lin et al. 2012). Similarly, other factors whose involvement in the metastatic process has been better described for other tumor types (integrins and the gene

COX2) were reported to contribute to the metastatic phenotype of chondrosarcoma cells *in vitro* (Tang 2012).

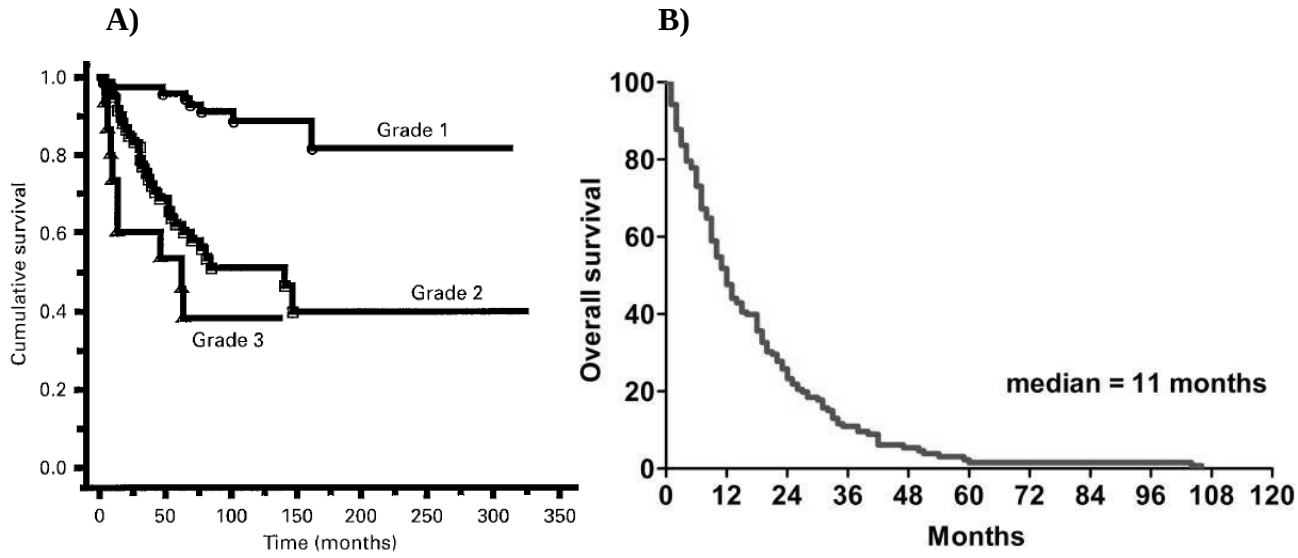


Figure 4: A) Cumulative survival rates according to chondrosarcoma grading. Figure adapted from (Fiorenza, Abudu et al. 2002). **B)** Overall survival for patients with unresectable chondrosarcoma tumors of mixed grades (1,2 and 3). Figure adapted from (van Maldegem, Gelderblom et al. 2014)

3.1.5 Chondrosarcoma treatment

3.1.5.1 Surgery

For all grades and subtypes of nonmetastatic chondrosarcoma, complete surgical tumor removal stands for the only therapeutic option (Figure 5). The chosen treatment generally is a wide, *en-bloc*, excision for all central malignant (Grade 2/3 and dedifferentiated) and peripheral chondrosarcoma tumors. For central low Grade 1 chondrosarcoma tumors, *curettage* is the usual treatment, combined with adjuvant therapy (for example, phenolization or cryosurgery with liquid nitrogen), then followed by filling the cavity with bone graft (Gelderblom, Hogendoorn et al. 2008).

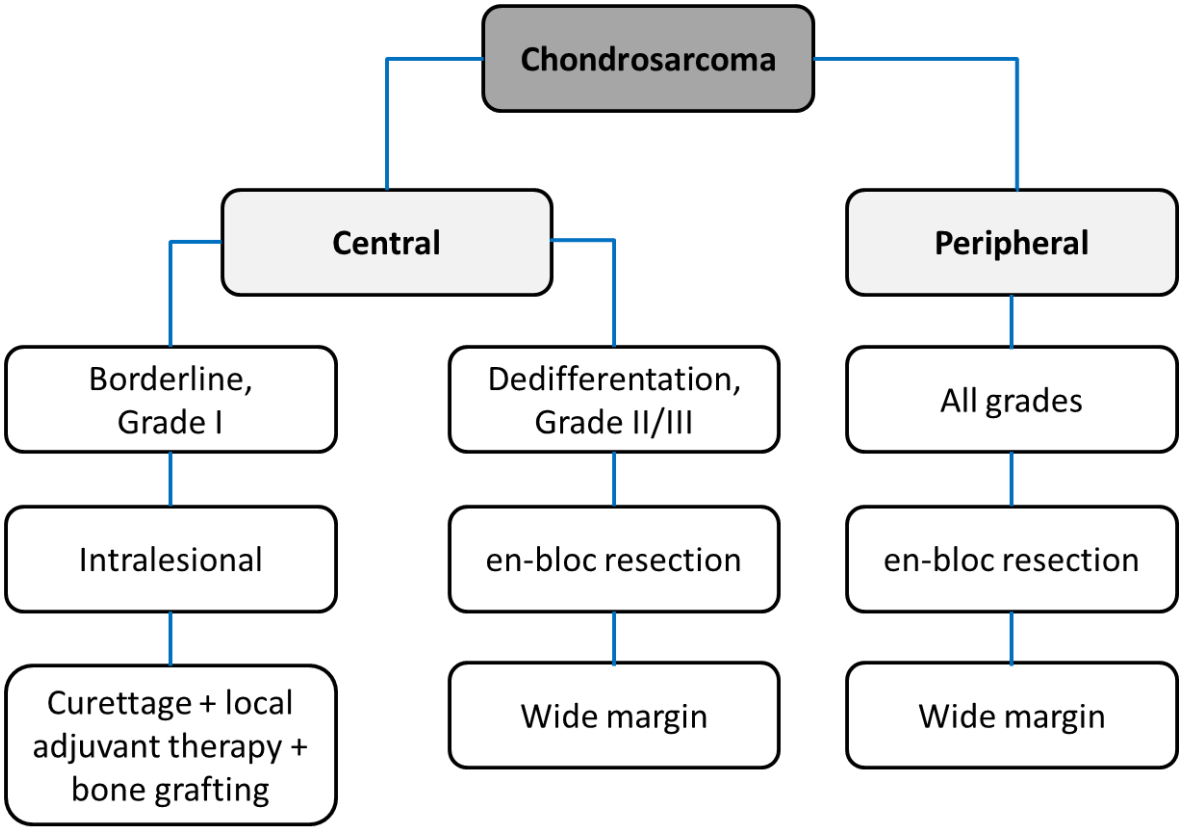


Figure 5: Flowchart of the surgical management of central and peripheral chondrosarcoma. Figure adapted from (Gelderblom, Hogendoorn et al. 2008)

3.1.5.2 Radiotherapy

Chondrosarcoma cells are slow-diving making them a complicated target for radiotherapeutic treatments partly explaining why chondrosarcoma tumors are highly resistant to these treatment modalities (Onishi, Hincker et al. 2011). Nevertheless, radiotherapy can be used in two different scenarios and usually for palliative treatment: when the tumor resection was incomplete during surgery and for unresectable cases (Gelderblom, Hogendoorn et al. 2008; Riedel, Larrier et al. 2009). Given the limited efficacy of previously tested radiotherapeutic regimens, there are currently various ongoing clinical trials testing novel therapeutic options for chondrosarcoma (NCT01182753, NCT00496522, NCT01346124, NCT01659203).

3.1.5.3 Chemotherapy

Systemic drug treatments have existed for many decades in single-drug or combined regimens and for certain cancer types they are extremely effective therapies. Similarly to radiotherapy, chondrosarcoma tumors are highly resistant to conventional chemotherapies (doxorubicin, cisplatin, gemcitabine, and others), reflected in poor outcomes when surgery is not curative in patients (Italiano, Mir et al. 2013). The cartilage architecture -poorly vascularized and with a high abundance of extracellular matrix components- very likely plays a role in chemoresistance as it is not a permeable tissue for drug delivery. Additionally, chondrosarcoma tumors are highly heterogeneous, and there is still a big debate about which cell types should mainly be targeted by drug treatments (David, Blanchard et al. 2011).

Nevertheless, most likely the cartilage niche is not the unique contributor to chemoresistance since chondrosarcoma cells *in vitro* can tolerate high doses of different chemotherapeutic drugs. In this regard, numerous works have tried to elucidate the possible molecular mechanisms behind this process. For example, it is well reported that the protein P-glycoprotein is highly expressed in chondrosarcoma. This protein is an ATP-dependent membrane pump which secretes drugs and other small molecules to the extracellular space. Importantly, 90% of all cartilaginous tumors are positive for P-glycoprotein immunostaining which makes them insensitive to cyclophosphamide, doxorubicin, vincristine, cisplatin, methotrexate and dacarbazine (Terek, Schwartz et al. 1998; Wyman, Hornstein et al. 1999). Moreover, there is evidence that chondrosarcoma overexpress several anti-apoptotic genes such as BCL-2 and survivin (also known as BIRC5), preventing cell death induced by conventional chemotherapy (Kim, Kim et al. 2009; Lechler, Renkawitz et al. 2011).

There are other possible mechanisms of drug resistance utilized by chondrosarcoma cells, such as expressing large amounts of telomerase, hypoxia inducible factor-1α (HIF-1α) or

cyclooxygenase-2 (COX-2) which could confer cellular immortality, generate new vasculature and promote inflammation, respectively (Onishi, Hincker et al. 2011).

3.1.5.4 Targeted therapies

Given the lack of efficacy of available treatments, there is an urgent need for the development of novel therapeutic approaches. Current research efforts are focused on targeting molecular factors that have been shown to be active or to contribute to chondrosarcoma pathogenesis and are listed below. Additionally, a brief summary of the clinical studies supporting the development of novel treatments in chondrosarcoma is presented in Table 2.

1) Isocitrate dehydrogenase

Recent studies have shown that isocitrate dehydrogenase 1/2 (IDH1/2) heterozygous mutations occur in various cancers, including chondrosarcoma (Amary, Bacsi et al. 2011). IDH1 or IDH2 are enzymes that catalyze the conversion of isocitrate to α-ketoglutarate (αKG), in a NAD(P)-dependent fashion, as part of the Krebs cycle. Mutated IDH1 and IDH2 enzymes produce d-2-hydroxyglutarate (2HG) from αKG conversion. It has been reported that for conventional chondrosarcoma approximately 50% of all tumors harbor IDH1/IDH2 mutations. In addition to this, in benign enchondromas IDH1/IDH2 mutations are present in 87% of cases, while for dedifferentiated chondrosarcoma in 54% of all cases. Interestingly, neither benign (osteochondromas) nor malignant (peripheral chondrosarcoma) superficial cartilaginous carry IDH1/IDH2 mutations (Schaap, French et al. 2013).

The accumulation of the 2HG metabolite generates various cellular changes, mostly through epigenetic regulation by causing hypermethylation of certain DNA regions by TET2

dioxygenase activation, which could potentially lead to carcinogenesis (Molenaar, Maciejewski et al. 2018). *In vitro* studies showed that treatment of cells with a IDH inhibitors drastically decreases the aberrant production of 2HG in chondrosarcoma cell lines, although is not yet clear if IDH inhibition affects cell proliferation and survival (Polychronidou, Karavasilis et al. 2017). Importantly, IDH1/2 mutations have been recently shown to be negative prognostic factors in chondrosarcoma patients (Lugowska, Teterycz et al. 2018).

2) The hedgehog pathway

As mentioned previously in section 3.1.3 (*Etiology*), the IHH and PTHrP signaling pathways are key players in the regulation of chondrocyte proliferation and differentiation. Therefore, it is not surprising that changes on the expression levels and/or mutations in any of the components of these pathways may be detrimental for bone development and eventually contribute to chondrosarcoma pathogenesis. In addition to this, high-grade chondrosarcoma tumors are known to express high levels of IHH factors (Tiet, Hopyan et al. 2006). By inhibiting the IHH pathways in chondrosarcoma, cells are less proliferative and migratory (Xiang, Jiang et al. 2014), and it was also shown *in vivo* that this IHH blockade causes tumor growth inhibition (Campbell, Nadesan et al. 2014).

Given the promising results observed in pre-clinical settings with IHH drug inhibitors, they were subsequently tested in two independent chondrosarcoma clinical studies. Unfortunately, none of them showed a clinical benefit (BusinessWire 2012; Italiano, Le Cesne et al. 2013). One possible explanation for the failure of both trials could be that both drugs tested (saridegib and vismodegib) are inhibitors of the IHH receptor *smoothen* (SMO), which might be constitutively active (gain-of-function mutation) regardless the overexpression of the HH ligand.

3) Receptor tyrosine kinases:

3.1) The PI3K–Akt–mTOR pathway

The phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway is constitutively activated in multiple cancer types, causing reduced apoptosis and enhanced cell proliferation. The pathway is activated by ligands that bind to receptor tyrosine kinases (RTKs), such as IGF-1R and PDGFR, that have been shown to be active in chondrosarcoma (Schrage, Briaire-de Bruijn et al. 2009; Zhang, van Oosterwijk et al. 2013). These studies demonstrate that RTK activation in chondrosarcoma is directly linked to the PI3K–Akt–mTOR signaling as RTK inhibitors diminish phosphorylation of downstream effectors (Akt and S6 kinase) resulting in chondrosarcoma cell growth inhibition *in vitro* and *in vivo*.

Additionally, mTOR pathway was shown to be important in chondrosarcoma and its blockade decreased the *in vitro* proliferation rate of cells and prevented tumor recurrence in a chondrosarcoma rat model (Perez, Decouvelaere et al. 2012). Combining mTOR inhibitors with chemotherapeutic agents has also been tested in chondrosarcoma. For example, chondrosarcoma patients with unresectable tumors who were treated with mTOR inhibitors plus cyclophosphamide showed promising disease control rates, which stimulated the development of an ongoing phase II trial of sirolimus with cyclophosphamide to study safety and efficacy (EudraCT: 2013-005155-32). A very recent phase II study testing the combined effect of doxorubicin (in a liposomal formulation) together with the mTOR inhibitor temsirolimus proved in roughly half of the enrolled patients a progression free survival of 315 days (Trucco, Meyer et al. 2018).

The activation of the PI3K–Akt pathway caused by IGF-1R is a known mechanism of mTOR-inhibitor resistance in cancer. Reduced AKT activation levels caused by combined regimens of IGF-1R and mTOR inhibitors in different studies stimulated the design of

multiple clinical trials to test such combinations in sarcoma patients with some promising results (Polychronidou, Karavasilis et al. 2017). Lastly, inhibition of PDGFR with imatininb has not shown encouraging results for the treatment of a cohort of patients with unresectable chondrosarcoma tumors (Grignani, Palmerini et al. 2011).

3.2) VEGF and angiogenesis

Tumor vascularization is a fundamental step in cancer development and in chondrosarcoma it has been shown to contribute to pathogenesis and tumor progression (Kubo, Shimose et al. 2013; Liu, Chen et al. 2014) and its inhibition demonstrated to potently inhibit tumor growth in a xenograft mouse model (Klenke, Abdollahi et al. 2007).

In addition to these results, when the angiogenesis mediator COX-2 (expressed in 2/3 of all chondrosarcoma tumors) is inhibited with the drug celecoxib, chondrosarcoma cell viability is affected *in vitro* and tumor growth is inhibited in chondrosarcoma xenografts (Schrage, Machado et al. 2010).

Currently there is an ongoing Phase II study (NCT01330966) assessing the clinical efficacy and safety of a broad RTK inhibitor, pazopanib, which has VEGF, cKIT and PDGFR among some of its targets. All enrolled patients have surgically unresectable chondrosarcoma tumors. A similar approach follows the ongoing Phase I/II trial (NCT02389244) by testing the multi-RTK inhibitor regorafenib in a cohort of patients with bone metastatic sarcomas.

4) *Histone deacetylases*

Epigenetic factors are strong modulators of gene expression in cells and regulate numerous cellular processes. It has been widely reported that their deregulation can contribute to the malignant cellular transformation of cells. Histone acetylates and deacetylases are epigenetic

factors responsible for activating or deactivating gene expression in certain genomic regions by changing the RNA polymerases accessibility to the chromatin (Sharma, Kelly et al. 2010).

In chondrocytes, numerous studies have shown that the regulation of histone acetylation/deacetylation is a very active process, underlying its relevance for normal cartilage homeostasis (Guo, Zhao et al. 2014; Li, Wei et al. 2014). Most importantly, direct inhibition of histone deacetylases (HDACs) inhibited the growth of chondrosarcoma cells by inducing cell cycle arrest and apoptosis and diminishing tumor growth *in vivo*, demonstrating that HDAC inhibitors can be promising drug therapies (Sakimura, Tanaka et al. 2007; Zhu, Gu et al. 2015). Currently, there is a Phase II clinical study testing the clinical efficacy of the HDAC inhibitor romidepsin in different metastatic and unresectable sarcoma types, including chondrosarcoma (NCT00112463).

5) *Immunotherapies*

It has recently been described that chondrosarcoma tumors are infiltrated by various types of immune cells (such as CD8+, CD3+, CD4+ and CD163+), suggesting that the immune system could play an important role in chondrosarcoma pathogenesis. For example, CD163+ macrophage depletion slowed down tumor progression in a Swarm chondrosarcoma rat model (Simard, Richert et al. 2016). In addition to this findings, a 2016 study demonstrated that PD-L1 and HLA class I expression accompanied with T-cell and macrophage tumor infiltration occurs in 50% of all dedifferentiated chondrosarcomas patients (Kostine, Cleven et al. 2016).

Given these results, there are currently two Phase II clinical studies assessing the clinical benefit of immune checkpoint inhibitors in chondrosarcoma. So far it has been reported that one of them, pembrolizumab, exhibited partial tumor remission in one out of the six patients enrolled with dedifferentiated chondrosarcoma (NCT02301039).

6) Other targeted therapies

The enzyme EZH2 is a protein that participates in the transcriptional repression through histone methylation of certain genes. In different cancer types, EZH2 has been found to be deregulated or mutated and to contribute to pathogenesis (Kim and Roberts 2016). In high grade chondrosarcoma cell lines, EZH2 was found to be highly expressed, and its depletion by the DZNep inhibitor caused cell death *in vitro (Girard, Bazille et al. 2014).* Similarly, in myxoid chondrosarcoma the EZH2 depletion also resulted in cell proliferation inhibition (Shen, Cote et al. 2016). There are currently two ongoing clinical trials testing the clinical efficacy of an EZH2 inhibitor (tazmetostat) in pediatric (NCT02601937) and adult (NCT02601950) patients with myxoid chondrosarcoma, a tumor type with no clear evidence of cartilaginous differentiation.

On the other hand, zoledronic Acid is also currently tested in a Phase 1B clinical trial study as neoadjuvant therapy (NCT03173976). In few words, zoledronic acid slows down bone resorption and produces beneficial effects on bone metabolism in cancer patients. In chondrosarcoma, it was already shown that zoledronic acid may have a positive impact in tumor-associated pain and also in improving patient quality of life, independently from other administered therapies and disease control (Montella, Addeo et al. 2009).

Drug Name	Study ID	Mechanism of action	Study Design	Study Population	Status
Targeting IDH mutations					
AG-221	NCT02273739	Oral IDH2 inhibitor	Phase I/II	Study of orally administered AG-221 in subjects with advanced solid tumors, including chondrosarcoma with an IDH2 mutation	Ongoing, recruitment completed
AG-881	NCT02481154	Oral IDH1/2 inhibitor	Phase I	Advanced solid tumors, including chondrosarcoma, with IDH1/2 mutations	Ongoing, recruitment completed
AG-120	NCT02073994	Oral IDH1 inhibitor	Phase I	Study of orally administered AG-120 in subjects with advanced solid tumors, with an IDH1 mutation	Ongoing, recruitment completed
Metformin + chloroquine	NCT02496741	Oral antidiabetic (metformin) and oral antimalarial	Phase 1B	IDH1/2-mutated Patients With glioma, intrahepatic cholangiocarcinoma or chondrosarcoma	Ongoing, recruiting
PI3-Akt-mTOR pathway and RTKs					
Everolimus	NCT02008019	mTOR inhibitor	Phase II	A phase II study of everolimus in patients with primary or relapsed chondrosarcomas (CHONRAD)	Suspended due to everolimus unavailability
Imatinib	NCT00928525	PDGFR	Phase II	Study to determine if imatinib is active in diseases - such as chondrosarcoma - expressing the receptor for PDGF	Ongoing, recruitment completed
Sirolimus + Cyclophosphamide	NCT02821507	mTOR inhibitor (sirolimus) + DNA crosslinker (cyclophospamide)	Phase II	Sirolimus and cyclophosphamide in metastatic or unresectable myxoid liposarcoma and chondrosarcoma	Ongoing, recruiting
Dasatinib	NCT00464620	multi-RTKs inhibitor	Phase II	Trial of dasatinib in advanced sarcomas, including chondrosarcoma	Completed
Angiogenesis					
Regorafenib	NCT02389244	VEGFR and other RTKs	Phase II	A study evaluating efficacy and safety of regorafenib in patients with metastatic bone sarcomas (REGOBONE)	Ongoing, recruiting
Pazopanib	NCT01330966	VEGFR and other RTKs	Phase II	Study of pazopanib in the treatment of surgically unresectable or metastatic chondrosarcoma	Completed
Gemcitabine +Pazopanib	NCT01532687	Nucleoside analog (gemcitabine) + VEGFR and other RTKs (pazopanib)	Phase II	Gemcitabine hydrochloride with or without pazopanib hydrochloride in treating patients with extraskeletal myxoid Chondrosarcoma	Ongoing, recruiting

Pazopanib	NCT02066285	VEGFR and other RTKs	Phase II	Trial of pazopanib in patients with solitary fibrous tumor and extraskeletal myxoid chondrosarcoma	Unknown
HDAC Inhibitors					
Romidepsin	NCT00112463	HDAC inhibitor	Phase II	Patients with metastatic or unresectable soft tissue sarcoma	Suspended due to disease progression
Immune Checkpoint Inhibitors					
Pembrolizumab	NCT02301039	Anti-PD1	Phase II	This is a multi-institutional phase II study of pembrolizumab in patients with advanced sarcomas. This study will have two treatment groups, one group for patients with soft tissue sarcoma and one group for patients with bone sarcoma.	Ongoing, recruitment completed
Nivolumab + ABI-009	NCT03190174	Anti-PD1 (Nivolumab) + mTOR inhibitor (ABI-009)	Phase 1B	This study investigates the safety/toxicity and potential anti-tumor activity of sequential administration of nivolumab and escalating doses of the mTOR inhibitor ABI-009 in advanced sarcomas	Ongoing, recruiting
Nivolumab + Ipilimumab	NCT02982486	Anti-PD1 (Nivolumab) + anti-CTLA4 (Ipilimumab)	Phase II	A phase II of nivolumab plus ipilimumab in non-resectable sarcoma and endometrial carcinoma	Not yet recruiting
Other therapies					
Tazemetostat	NCT02601950	EZH2 inhibitor	Phase II	A phase 2 study of the EZH2 inhibitor tazemetostat in adult subjects with relapsed or refractory INI1-negative tumors or synovial sarcoma	Ongoing, recruiting
Tazemetostat	NCT02601937	EZH2 inhibitor	Phase I	A Phase 1 study of the EZH2 inhibitor tazemetostat in pediatric subjects with relapsed or refractory INI1-negative tumors or Synovial Sarcoma	Ongoing, recruiting
Zoledronic Acid	NCT03173976	Anti-Osteoclast Therapy	Phase 1B	Clinical trial assessing the safety and efficacy of neoadjuvant zoledronic acid in patients with resectable any grade chondrosarcoma	Ongoing, recruiting

Table 2: Ongoing and recently finished clinical trials in various chondrosarcoma subtypes. Adapted from (Polychronidou, Karavasilis et al. 2017) and (Brown, Schiavone et al. 2018)

Although the conventional approaches for the development of novel therapeutic options has not far succeeded in chondrosarcoma, the field is moving rapidly, which is echoed in the growing number of clinical studies. Nevertheless, given this unmet need, new alternatives for the discovery of molecular targets must be exploited. In this regard, microRNAs have been widely shown as key contributors to cancer development and progression and, additionally, as sensitive and specific biomarkers for certain cancer types. Thus, the identification of relevant microRNAs in chondrosarcoma may represent a crucial path for the identification of molecular drivers of therapeutic and diagnostic value.

3.2 microRNAs

microRNAs (miRNAs) are small non-coding RNAs of ~20-30nt in length that modulate gene expression. These molecules were firstly characterized in 1993 by Ambros and colleagues as endogenous regulators of genes in *Caenorhabditis elegans* (Lee, Feinbaum et al. 1993). Although there are exceptions, the vast majority of miRNAs are transcribed from precursor-primary miRNA molecules (pri-miRNA) in defined genomic regions, which then are further processed into the mature miRNAs, as it will be discussed in more detail on the following section (*miRNA biogenesis and regulation*) .

As a general rule, mature miRNAs modulate gene expression by base-pairing with complementary specific regions within the 3'UTR sequence of a given gene, causing mRNA expression inhibition (Bartel 2004). Nevertheless, it is also known that this interaction can occur at the 5' UTR, although much less frequently (Lee, Ajay et al. 2009). Additionally, it has been demonstrated that under certain circumstances a minority of miRNAs can enhance gene expression of mRNAs, instead of repressing it.

miRNAs play a key role in animal development and they are also involved in virtually every existing biological process within cells, such as proliferation, differentiation, apoptosis, and many others. Because of their strict involvement in cell and tissue homeostasis, deregulation of miRNAs levels can contribute to different human diseases, such as cancer (Alvarez-Garcia and Miska 2005). Consequently, miRNA-based therapeutic strategies have been developed in order to restore the normal levels of deregulated miRNAs (Krutzfeldt, Rajewsky et al. 2005; Christopher, Kaur et al. 2016).

Lastly, miRNAs are found in distinct body fluids as extracellular miRNAs, either associated with proteins or within small vesicles, and they are part of a sophisticated mechanism of cell-to-cell communication (Sohel 2016).

3.2.1 *miRNA biogenesis and regulation*

The synthesis of miRNAs is complex multistep process dependent on a RNA polymerase II transcription occurring in the cell nucleus (Lee, Kim et al. 2004). Most of miRNAs are located within intronic regions of protein-encoding genes whereas a small minority within exons. Very commonly, these intragenic miRNAs regulate the expression levels of the gene(s) that they are "embedded" in. The remaining miRNAs –those which are not located within genes, and are therefore known as intergenic- are transcribed independently, as they have their own promoter regions. Although there are multiple alternative ways in which cells synthesize miRNAs, the dominant one is the canonical biogenesis pathway. This pathway (described in detail in Figure 6) consists of the initial transcription (by RNA polymerase II) of a pri-miRNA precursor of 1-3 kb which is immediately processed in the nucleus by the Drosha/ DGCR8 complex into a ~70 nt pre-miRNA molecule with a double-stranded hairpin loop region in its structure. These pre-miRNAs are then transported to the cytoplasm by a nuclear export protein named Exportin 5. Once in the cytosol, the Dicer/TRBP complex excises the pre-miRNA into ~22 bp double-stranded miRNA structures (miRNA/miRNA*) which are afterwards separated into two mature miRNAs. One of these strands is degraded based on thermodynamic stability of the two ends of the RNA duplex (Schwarz, Hutvagner et al. 2003) and only one mature miRNA remains. The latter one is loaded into Argonate (AGO) to form the RNA-induced silencing complex (RISC).

Similarly to protein-encoding genes, miRNA transcription can be regulated by different effectors. The expression of miRNA genes in close proximity to CpG islands can be affected by methylation (Han, Witmer et al. 2007). Other mechanisms of miRNA expression do exist, for example, a small fraction of them are controlled by circadian rhythm regulation (Cheng, Papp et al. 2007). Several other processes mediate miRNA abundance at a posttranscriptional

level, such as miRNA edition, target transcript abundance ("sponging") or by changes generated by specific factors during miRNA biogenesis (Cai, Yu et al. 2009; Gebert and MacRae 2019).

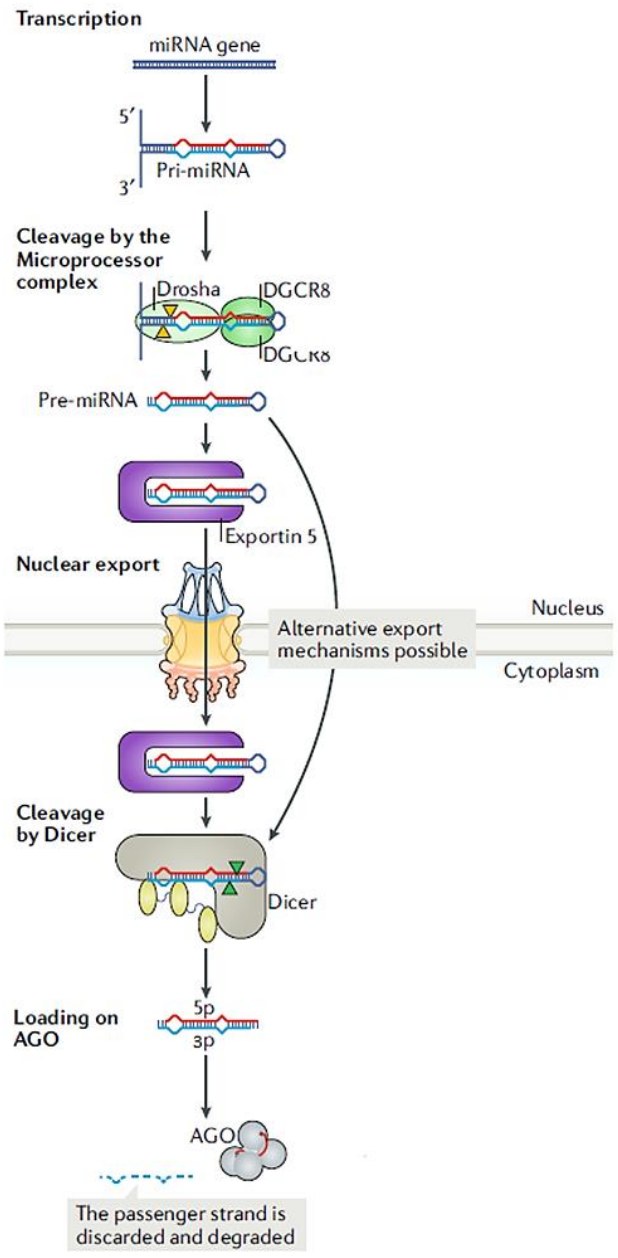


Figure 6: The canonical pathway is the principal pathway for miRNA synthesis. Here, pri-miRNAs (1-3kb long) are transcribed by RNA Pol II from their genes and then excised to pre-miRNAs by the DGCR/Drosha complex. DGCR8 recognizes a specific sequence motif within the pri-miRNA while Drosha (an RNAse III enzyme) cuts the pri-miRNA duplex at the start of the hairpin structure giving a pre-miRNA as product. The transport of pre-miRNAs towards the cytoplasm is mediated by the protein Exportin 5 in a GTP-dependent fashion. In the cytoplasm, the enzyme Dicer removes the terminal loop of the pre-miRNA generating as a product a dsRNA duplex, which can be loaded into Argonaut (AGO1-4, in humans) in an ATP-dependent way. The orientation of each strand gives the name of the mature miRNA (either 5p or 3p). One strand will remain with AGO (guide strand) whereas the other one (passenger strand) will be degraded, a process subjected to cell type and environmental factors, ranging from equal proportions to one strands predominating over the other one. Figure adapted from (Gebert and MacRae 2019)

3.2.2 *miRNA gene-mediated regulation*

As already mentioned, the dominating gene expression mechanism mediated by miRNAs involves the direct interaction between certain residues of a mature miRNA with the 3' UTR of a particular mRNA target transcript, resulting in gene expression inhibition (miRNA-mediated upregulation of a target can also occur, but it is extremely rare). Additionally, miRNAs may also bind to 5'-UTR regions of genes, but their frequency of occurrence is very low.

Interestingly, bioinformatic analysis on the conserved miRNA binding sites within mRNAs suggests that potentially 30-60% of all human genes are regulated by miRNAs (Lewis, Burge et al. ; Friedman, Farh et al. 2009). In this regard, there are three main mechanisms that cells use to silence target genes by miRNA direct regulation. One of them is the site-specific cleavage via RISC which, as noted in section 3.2.1, involves the protein AGO bound to a mature miRNA. The specificity of the RISC complex against an mRNA target depends on the degree of complementarity between the miRNA seeding sequence and the UTR, also known as the miRNA response element (MRE). If the degree of complementary in a given miRNA:MRE is high (full complementarity), what will happen is that the mRNA will be cleaved by RISC. On the other hand, if there is not full complementarity between the miRNA:MRE (as it happens in the majority of cases in animal cells) AGO will not exert its endonuclease activity over the mRNA transcript. Instead, there will be a non-cleavage mRNA repression, either by mRNA destabilization or translational repression (Figure 7). The first needs the recruitment of deadenylating proteins (also known as PABP, which remove mRNA poly-A tails) and decapping proteins towards the RISC complex, resulting in the destabilization and degradation of the target mRNA (O'Brien, Hayder et al. 2018). Inhibition of translation is a less well understood phenomenon (probably due to the presence of

numerous mechanisms) that possibly occurs by RISC preventing the formation of a circular complex, which is necessary for the correct assembly of the ribosome to initiate protein synthesis. Regardless the mechanisms for miRNA-dependent gene expression inhibition, the final product is a downregulation of the protein encoded by the target gene.

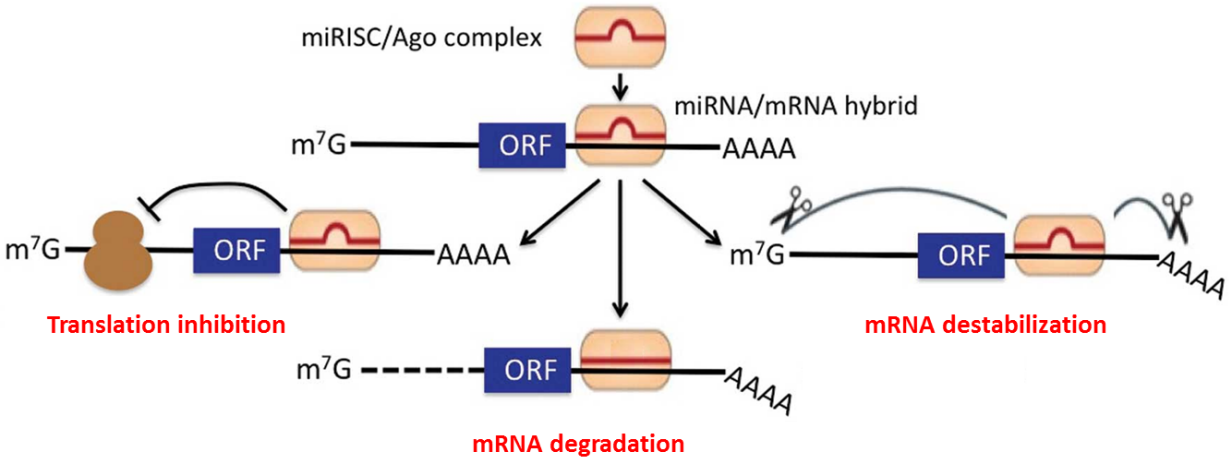


Figure 7: Representative illustration of the different molecular mechanisms for miRNA-mediated gene expression inhibition. ORF = open reading frame. Adapted from (Piva, Spandidos et al. 2013)

3.2.3 *miRNA clusters and families*

miRNAs can be transcribed individually or as a part of a long polycistronic transcript referred to as miRNA cluster. These are frequently composed of two or three miRNAs (although clusters with 6 or more miRNA genes exist) and coordinate complex regulatory mRNA networks and fine-tune the regulation of multiple biological processes. Members of the same cluster do not necessarily have similarities in their sequences (Aravin, Lagos-Quintana et al. 2003), and given their high degree of evolutionary conservation, it is very likely that many clusters were generated by *de novo* duplication of a miRNA. This could have conferred an adaptive advantage to the harboring cell, as multiple miRNAs exert a more robust regulation than individual miRNAs (Wang, Luo et al. 2016). When miRNAs from a cluster have similar

seed sequences -thus they share similar mRNA targets- we call them miRNA families. It is worth mentioning that is not exclusive for members of a miRNA family to be produced from a same transcript as they can originate from different chromosomes and still be part of the family as long as they share a high degree of sequence homology, at least in the seeding region (the miR-200 family is a well-studied case) (Tanzer and Stadler 2004).

Importantly, mutations in miRNAs or aberrant regulation of miRNA production often lead to the development of diseases, such as cancer (Ardekani and Naeini 2010).

3.3 miRNAs in cancer

The deregulation of one or more miRNAs can have severe negative consequences in human cells. The involvement of miRNAs in cancer was firstly reported on 2002 (Calin, Dumitru et al. 2002) and since then the field has grown exponentially (>35,000 PubMed hits as of March 2019). Numerous miRNAs are nowadays known to contribute to different stages of cancer pathogenesis, such as metastasis, resistance to chemo and radiotherapy, cell proliferation, EMT, etc. (Peng and Croce 2016). miRNAs that are over-expressed in cancers may function as oncogenes (and therefore are called oncomiRs), whereas downregulated may indicate a tumor suppressor function (Lee and Dutta 2009).

Studying the role of miRNAs in cancer can be challenging. On the one hand, most phenotypic traits are regulated by more than just one miRNA, and these may not be related or even similarly deregulated. On top of this, tumors are heterogeneous by nature and not all cell types within a tumor might suffer from miRNA deregulation in the same way. One must also take into account that miRNA function in oncogenesis can also be context dependent (subjected to the differentiation status of cells, for example). Secondly, one miRNA can regulate numerous gene targets -and one target can be regulated by many miRNAs- so

deciphering the main contributors of these complex networks is a key point towards understanding the role of a given miRNAs in the disease.

In tumors, downregulated miRNAs are more abundant than upregulated ones (Lu, Getz et al. 2005). There is a broad array of mechanisms behind miRNA downregulation. One of them is the transcriptional silencing caused by hypermethylation at miRNA regulatory regions. Interestingly, many miRNA loci associated with CpG islands have been reported to be sensitive to methylation status (Weber, Stresemann et al. 2007). Additionally, roughly half of human miRNAs are located within fragile genomic regions, suggesting that the downregulation can also occur due to genomic loss of those DNA regions (Calin, Sevignani et al. 2004). Germline mutations within the miRNA sequence (especially on the seeding region) can affect drastically the thermodynamic interactions bridging miRNA:mRNA parings. It is also possible that the mutations in the seeding region of a miRNA can affect the regulation of new genes that were not targeted in the wild type condition. Moreover, mutations or deregulation in genes encoding miRNA-biogenesis machinery (such as Drosha or Dicer) have also been shown to be deleterious and to contribute to cancer (Kumar, Lu et al. ; Thomson, Newman et al. 2006). Lastly, transcription factors can also bind to miRNA promoter regions and reduce their expression (Jansson and Lund 2012).

On the other hand, upregulation usually occurs due to epigenetic regulation, activation mediated by transcription factors or by duplication of miRNA-encoding DNA regions (Peng and Croce 2016).

3.3.1 *miRNAs in chondrosarcoma: the unexplored field*

When compared to other types of sarcoma, such as osteosarcoma, the biological relevance of miRNAs in chondrosarcoma is still far from being well characterized (the Pubmed search

"miRNA + chondrosarcoma" gives <60 hits whereas the number is ~1,000 for osteosarcoma, as for March 2019). On top of this, most of the published studies included only limited *in vitro* functional data, without addressing *in vivo* functionality.

To the present date, there is limited global miRNA data from cancerous chondrogenic tissues. Past efforts have positively contributed to a better characterization of miRNA signatures in tumors of the cartilage. However, they have some limitations as they either included only a small number of chondrosarcoma clinical samples (and all with the same grading) (Sui, Liu et al.) or they chose almost-obsolete and less precise discovery approaches (Yoshitaka, Kawai et al. 2013). Ergo, these studies have not represented a very prolific source of candidate miRNAs that might be relevant for chondrosarcoma progression. Instead, several of the studied miRNAs in chondrosarcoma have possibly been chosen for being relevant in other cancer types or malignancies of the cartilage (i.e. arthritis).

Many individual deregulated miRNAs have been linked to chondrosarcoma aggressiveness (Li, Wang et al. 2015; Sun, Charbonneau et al. 2015; Bao, Ren et al. 2017), angiogenesis (Liu, Chen et al. 2014; Huang, Chang et al. 2016) and therapy resistance (Zhu, Wang et al. 2014; Tang, Zheng et al. 2016). Moreover, other studies have pointed out the involvement of miRNAs in chondrogenesis and have identified targets that are known to be deregulated in chondrogenic tumors, such as the master chondrogenic factor SOX9 and VEGF (Yoshitaka, Kawai et al. 2013; Li, Wang et al. 2015; Sun, Charbonneau et al. 2015). Interestingly, none of the published studies on miRNAs in chondrosarcoma have taken miRNA clusters or families into account.

3.3.2 *Circulating miRNAs*

A large volume of data indicates that small non coding RNAs (miRNAs) can greatly facilitate the right clinical characterization of tumors and their tissue of origin (Rosenfeld, Aharonov et al. 2008). In sarcomas, miRNA profiling in tumor tissue was shown to be a potent tool for tumor subtype classification, as well as for the assessment of aggressiveness and response to therapy (Subramanian, Lui et al. 2008; Renner, Czwan et al. 2012; Boro, Bauer et al. 2016; Wiemer, Wozniak et al. 2017).

Importantly, miRNAs can also be found in different body fluids, such as serum and plasma (Chen, Ba et al. 2008). Several studies have demonstrated that in the bloodstream, miRNAs are highly stable. When naked miRNAs are exogenously spiked into the bloodstream, they get immediately degraded, suggesting that endogenous miRNAs can resist RNAse degradation (Mitchell, Parkin et al. 2008). Nowadays, we know that this protection comes from the association of miRNA with proteins or for being packed within extracellular vesicles of different sizes (Figure 8). Although is not entirely clear how it occurs, circulating miRNAs can mediate cell-to-cell communications (Creemers Esther, Tijsen Anke et al. 2012). Importantly, thanks to recent technological advances, circulating miRNAs can be rapidly and accurately quantified by different affordable methodologies (Schwarzenbach, Nishida et al. 2014). One important technical aspect to take into consideration when measuring circulating miRNA levels is to normalize their expression against endogenous and exogenous (spike-in) reference miRNAs Endogenous “housekeeping” circulating miRNA are disease specific and have to be determined empirically (Schwarzenbach, da Silva et al. 2015).

Taken all these aspects into account, the potential of circulating miRNAs as blood-based non-invasive biomarkers is huge, especially in fields like sarcoma, where conventional biomarkers have so far failed to provide accurate diagnostic and prognostic uses (see Section

3.1.2.1, *Grading of chondrosarcoma tumors and its challenges*). In osteosarcoma, there is a growing number of publications addressing single or combined miRNA signatures form liquid biopsies aiming to improve the diagnostic accuracy of the disease (Raimondi, De Luca et al. 2017; Wang, Ning et al. 2017). On the other hand, in chondrosarcoma the amount of research focused on circulating miRNAs is alarmingly low. In fact, the very first article including circulating miRNAs from chondrosarcoma patients was published during the time that this thesis was being written (March, 2019) (Asano, Matsuzaki et al. 2019). For all these reasons, we believe that miRNAs, and especially circulating miRNAs, hold great promise for the discovery of novel and clinically helpful biomarkers in chondrosarcoma. Additionally, other than just being used for tumor subtype assessment, tumor-derived miRNAs could be evaluated to monitor complete tumor removal or tumor relapse in patients (Fuji, Umeda et al. 2019).

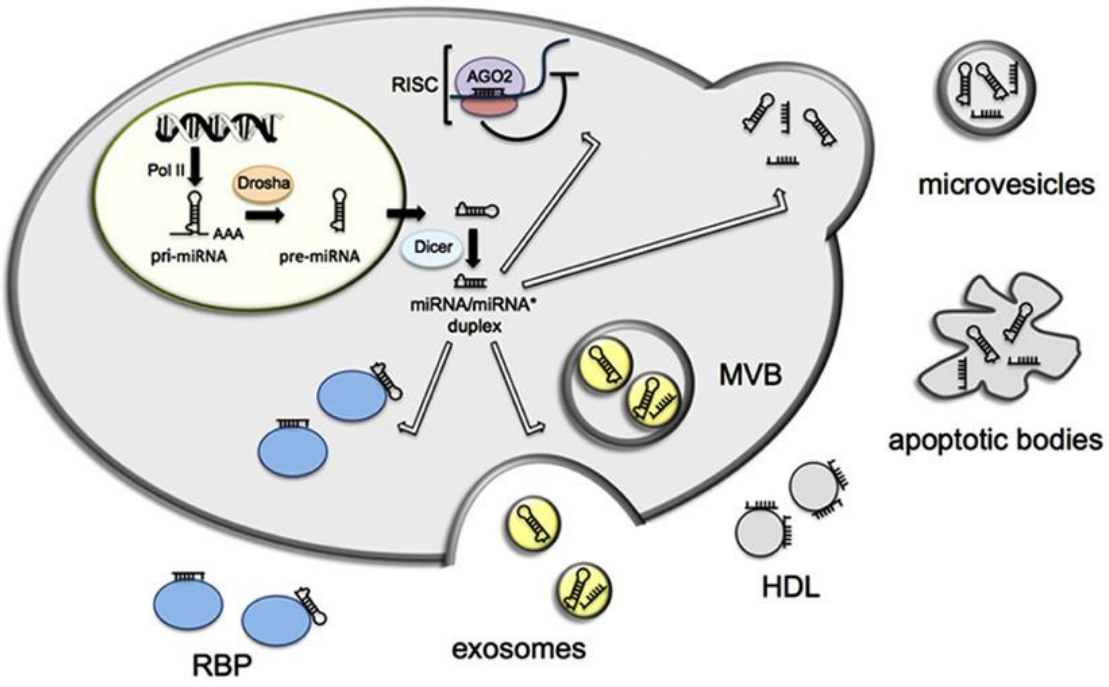


Figure 8: Schematic representation of the possible release mechanisms of miRNAs to the extracellular space. In the cytoplasm, miRNAs can be packed within extracellular vesicles which are actively secreted by cells, such as exosomes (40–120 nm) and microvesicles (0.1–1 μm). Apoptotic bodies (1–5 μm) are byproducts of apoptotic

cells and they also can contain miRNAs in their lumen. MiRNAs can also circulate in a microparticle-free fashion, either in association with high-density lipoproteins (HDL) or bound to RNA-binding proteins (RBP, such as AGO). When cells die, these miRNAs may be released passively, but also actively in living cells through protein channels by mechanisms which are still not clear (Kinet, Halkein et al. 2013).

4 Aim of the PhD thesis

Understanding the molecular mechanisms behind chondrosarcoma progression is of utmost importance for the development of targeted therapies. miRNAs play an essential role in cancer progression and uncovering their biological contribution and targets is a valuable tool for the discovery of novel cancerous genetic drivers. Thus, the **first sub-aim** of the present thesis was to identify deregulated miRNAs –with a focus on miRNAs that belong to the same cluster or family- in a cohort of human tumor samples. This included chondrosarcoma specimens of different grades as well as benign chondrogenic tumors that can progress towards chondrosarcoma.

The **second sub-aim** of this work focused on studying the phenotypic contributions of the chosen miRNAs to chondrosarcoma malignancy *in vitro*. Furthermore, we wanted to test if our miRNA candidates would also have an *in vivo* effect. In order to do this, it was necessary to firstly address a **third sub-aim**, being the establishment of a suitable *in vivo* orthotopic chondrosarcoma xenograft model. We tested and compared two-previously reported orthotopic mice models in chondrosarcoma based on various parameters, such as tumor penetrance, time until tumor initiation and metastatic spread. The **fourth sub-aim** consisted on performing an *in vivo* study with the chosen orthotopic model for the assessment of the effect of the selected miRNAs on tumor growth and metastasis formation.

As miRNAs exert their biological function by regulating other genes, our **fifth sub-aim** consisted on studying which miR-143/145 direct target(s) are affected from their deregulation, and how they (may) contribute to chondrosarcoma malignancy. Lastly, our final and **sixth sub-aim** addressed the following question: Could blood-circulating miRNAs be used as diagnostic biomarkers in chondrogenic neoplasms?

The results obtained from the third sub-aim are presented in section 5.1 “Additional Studies”, whereas all other sub-aims together are introduced in section 5.2 “Manuscript”.

5 Results

5.1 Additional Studies: Establishment of an Orthotopic Chondrosarcoma Mouse Model

Purpose

To establish a suitable chondrosarcoma xenograft mouse model to study the *in vivo* effect of the selected miRNAs in tumor growth and metastatic disease. For this, we tested two reported orthotopic xenograft mouse models in bone sarcomas.

Introduction

The main advantage of orthotopic cell injections is that they reproduce the original site for the development of cancer (Jacques, Renema et al. 2018). For being the best described chondrosarcoma models, we aimed to test the intra-osseous (intratibial) and para-osseous (paratibial) model.

In the paratibial model, cancer cells are injected next to tibia. Before that, the periosteum is briefly activated by gently scratching its surface with a needle. On the other hand, the intratibial model is based on injecting the cancer cells into the intramedullary cavity. For that, it is firstly required to generate a hole with a 21–26G needle in the tibial plateau to give access to a Hamilton needle to deliver the cells inside the medullary cavity (Figure 1).

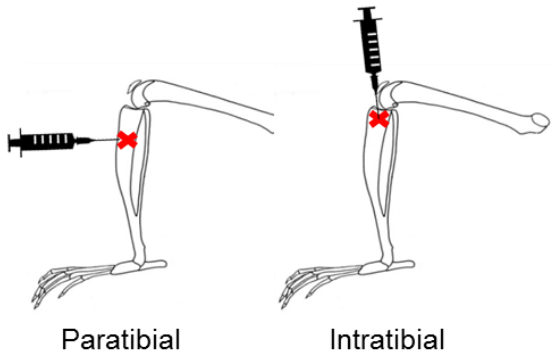


Figure 1: Illustration of the paratibial and intratibial injection approaches. Image adapted from (Uluckan, Segaliny et al. 2015)

Experimental design

The *in vivo* study was conducted with the approval of the Ethics Committee of the Canton Zurich Veterinary Office, and in accordance with the Swiss Animal Protection Law. Immunocompromised 6- to 7-week-old female SCID/CB17 mice were obtained from Charles River Laboratories 14 days before tumor cell injection (TCI). Two different cells amounts were injected for both tested cells lines (JJ012 and SW1353): 100,000 and 400,000 cells. The volume of injection in the intratibial model was 10µL (PBS/0.05% EDTA), whereas for the paratibial was 50µL (1:1 PBS/0.05% EDTA and Matrigel). The number of animals per each cell line/cell number condition was n=6 for the intratibial and n=7 for the paratibial model.

Tumor growth was monitored by caliper measurements and X-ray imaging on a weekly basis. All mice were sacrificed before they reached a maximum tumor volume of 300 mm^3. Additionally, as cell lines were equipped with a β-galactosidase gene (Figure 2), we stained different organs (lungs, liver, spleen, etc) *ex-vivo* with X-Gal to determine the quantity of micro and macrometastases as done previously (Gvozdenovic, Arlt et al. 2013).

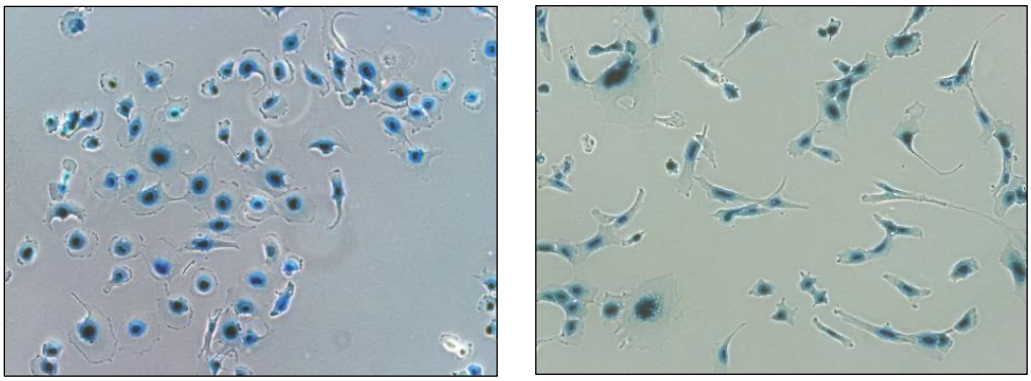


Figure 2: Pictures of JJ012 (left) and SW1353 (right) LacZ positive cells after fixation and X-gal staining (40X magnification)

Results

Tumor Penetrance

In the intratibial model, we observed a direct effect of the number of injected cells on the tumor penetrance rates (16.6% and 83.3% for 100,000 and 400,000 JJ012_LacZ injected cells, respectively) (Table 1). We only tested 400,000 cells paratibially as this model is less restrictive compared to the intratibial in terms of the maximum volume that can be injected due to limited space in the intramedullary cavity. For the same amount of injected cells the paratibial and intratibial models showed similar tumor penetrance efficiencies (400,000 cells, 85.7% and 83.3%, respectively).

Importantly, no tumors developed during the entire length of the study (4 months) in mice that with SW1353_LacZ cells. Previously reported reports have demonstrated that SW1353 can efficiently develop tumors subcutaneously (Jacques, Renema et al. 2018). Two other published works also showed that SW1353 cells can grow intratibially. However, there are some differences in the technical approach that might explain the discrepant findings. In one of the published studies, cells were previously cultured with Matrigel and then transplanted inside the tibia of SCID mice (Horas, Kurth et al. 2012). In the other case holes of 0.35mm in diameter where generated in the tibia with a drill, followed by flushing out the bone marrow cells in order to create more space for SW1353 cells to grow (van Oosterwijk, Plass et al. 2015). Although it is very unlikely, the retroviral transduction of SW1353 used in our study to generate SW1353 might have had an effect on their ability to form tumors *in vivo*. Noteworthy, the *in vitro* characterization of LacZ-tagged cells did not detect any differences when compared to the parental cell lines (data not shown). This goes in fully agreement with a study conducted by our group demonstrating that LacZ expression had no influence on the growth of tumors and metastasis formation in osteosarcoma (Arlt, Banke et al. 2011). To our

knowledge, there are no published studies that tested the ability of SW1353 cells to grow paratibially. The table below summarizes the tumor penetrance values obtained for all the conditions tested.

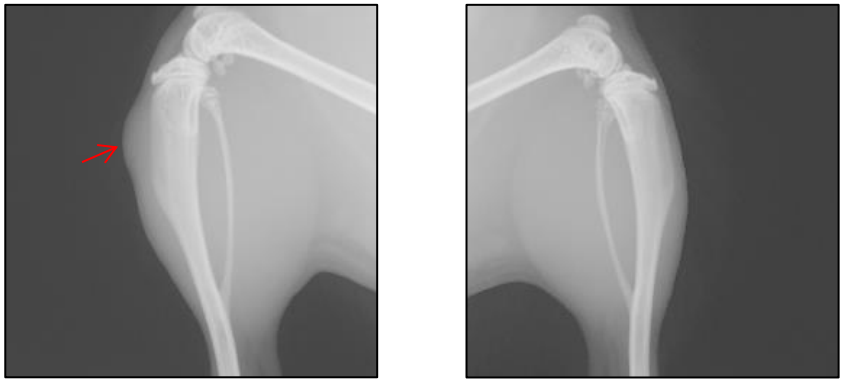
Cell line	N cells	Intratibial (%)	Paratibial (%)
JJ012_LacZ	100k	16.67	-
	400k	83.33	85.71
SW1353_LacZ	100k	0	0
	400k	0	0

Table 1: Summary of the tumor penetrance rates for different chondrosarcoma cells lines injected intratibially or paratibially in SCID mice

Times until tumor appearance

As it will be described in the next result section (5.2), artificial over-expression of miRNAs in chondrosarcoma cells was aimed to be performed in a doxycycline-inducible fashion. Therefore, to be able to start the induction of all mice simultaneously, it was crucial for our model to develop tumors within consistent time periods. Tumor detection was done by X-ray imaging and caliper measurements (Figure 2). Interestingly, mice injected with JJ012_LacZ cells paratibially showed both, shorter latency times until tumor appearance and better intragroup homogeneities compared to those injected intratibially (Figure 2.A and 2.B).

A)



Tumor-bearing leg Control leg

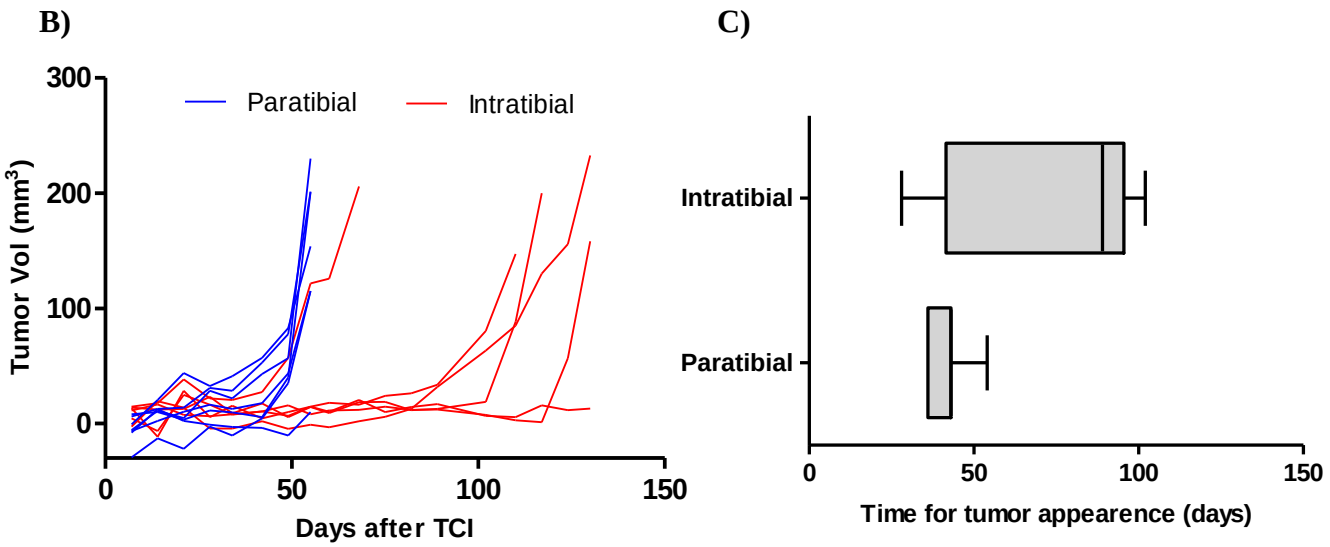


Figure 1: A) X-ray images in both tumor-bearing and control legs at time of tumor appearance (red arrow). **B)** Tumor growth monitored weekly for both models after tumor cell injection (TCI). **C)** Time until tumor detection in both tested models.

Tumor and metastasis features

Paratibial and intratibial tumors exhibited some typical clinical features of chondrosarcoma, such as osteolytic lesions in the bone (Figure 3.A). Tumor size doubled every week on average. Importantly, in both models, metastatic lesions were found in the lungs. Not surprisingly, all primary tumors were positively stained by X-gal (Figure 3.B). Other organs were tested for the presence of metastasis (spleen, liver and heart) but no metastatic lesions were found.

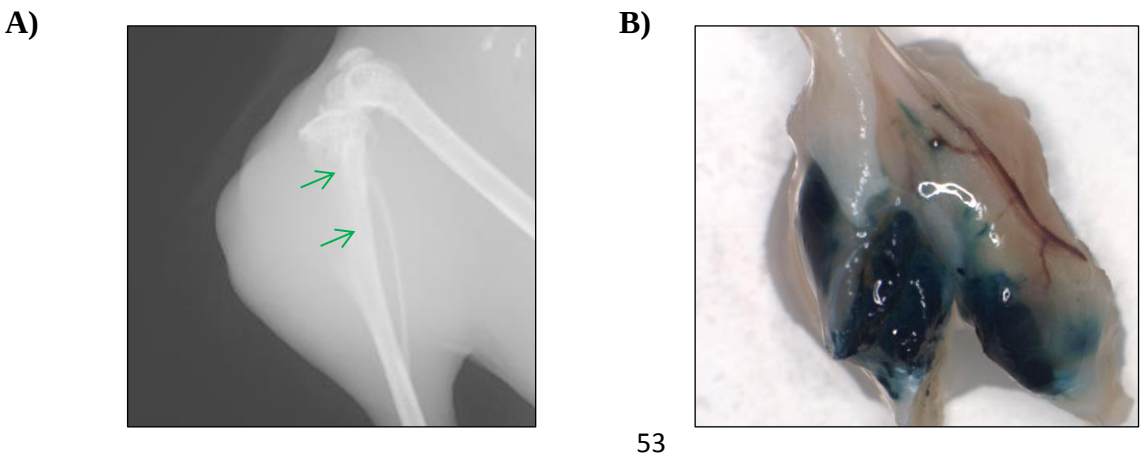
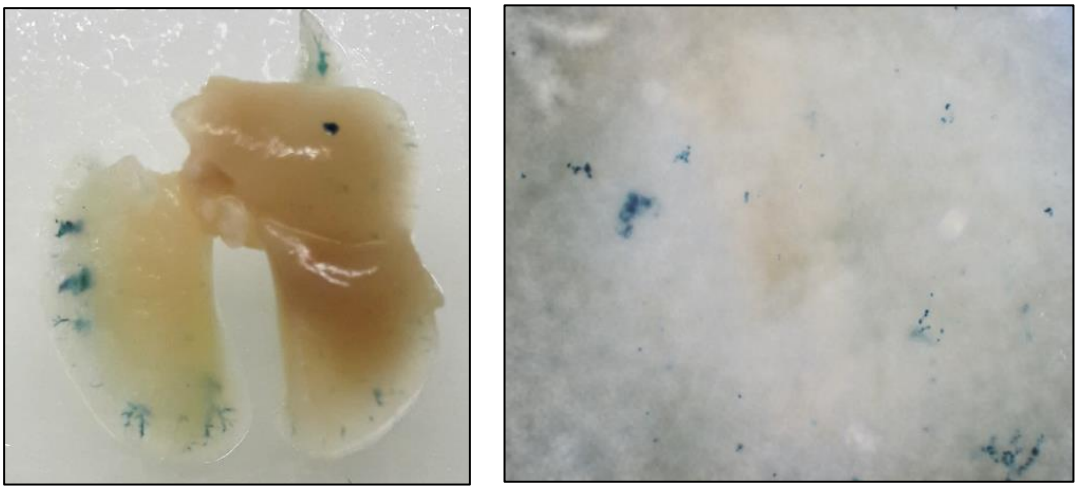


Figure 3: A) Osteolytic lesions (green arrows) seen by X-ray in both tested models (the picture corresponds to a paratibial case). **B)** Primary tumor obtained from a mouse injected intratibially and subsequently stained for X-gal. Blue areas represent tumor cells bearing the β-galactosidase (LacZ) gene.

Interestingly, intratibial tumors showed a higher metastatic potential than paratibial ones. Macrometastases were abundant in the lungs of intratibially injected mice, while rare in paratibially injected animals. Similarly, the amount of micrometastases was superior in the intratibial model (Figure 4).

A)

Intratibial



B)

Paratibial

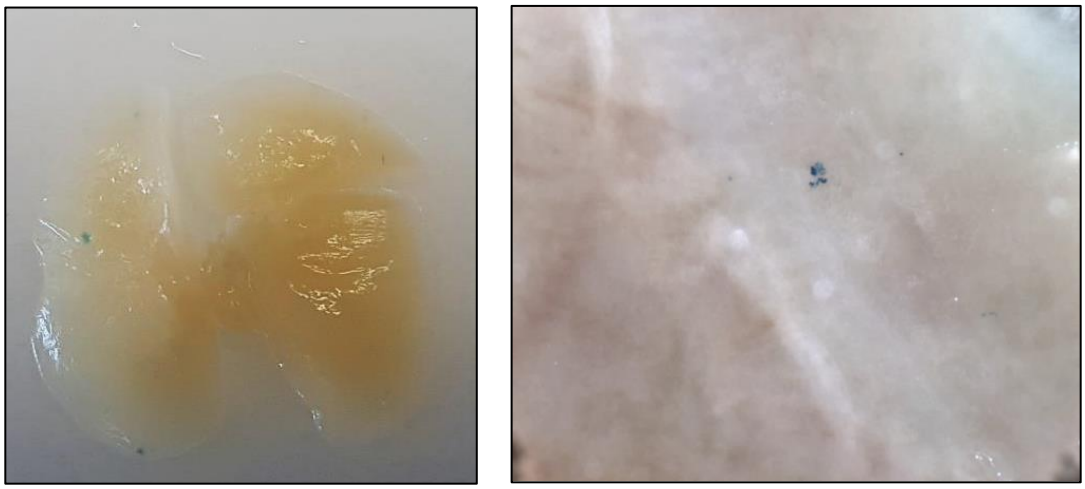


Figure 4: A) X-gal stained lungs showing macrometastases (left) or micrometastasis (right) in the lungs of mouse intratibially injected with chondrosarcoma cells **B)** X-gal stained lungs showing macrometastases (left) or micrometastasis (right) in the lungs of mouse paratibially injected.

Conclusions

The present study aimed to compare different orthotopic xenograft chondrosarcoma mouse models. We studied two models that are commonly used in the field of bone sarcomas, being intratibial and paratibial. Additionally, we evaluated two different human established chondrosacoma cell lines, SW1353 and JJ012, and in two different conditions: 100,000 and 400,000 injected cells.

Because SW1353 cells did not show signs of growth in any of the conditions tested (even several months after the tumor cell injection day), we disregarded them for following studies.

Taken together, from our work we can conclude that the paratibial model performed better than the intratibial in terms of homogeneity of tumor initiation times, which was a fundamental factor for the experimental setup to the subsequent study regarding the *in vivo* role of miRNAs in chondrosarcoma. Additionally, from a technical standpoint of view, paratibial injections are easier to apply and they are less painful for the animals. We believe that cells injected intratibially possibly require creating space in the bone cavity in order to grow, resulting in longer periods of time for tumors to develop.

For the same amount of injected cells, both injection approaches showed similar tumor penetrance rates, suggesting that JJ012 cells can grow well *in vivo*. In addition, the intratibial model is more suitable to study the metastatic potential of tumors, for forming more and larger metastatic lesions. We believe that for being injected directly in the medullar cavity, cancer cells have a better access to vasculature and, therefore, metastasize more efficiently.

In conclusion, we selected the paratibial model (400,000 JJ012 injected cells) for showing good penetrance rates and, importantly, very regular times of tumor appearance, a fundamental factor for the subsequent *in vivo* experiment presented in the result section 5.2.

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5.2 Manuscript: The miR-143/145 Cluster, a Novel Diagnostic Biomarker in Chondrosarcoma, Acts as a Tumor Suppressor and Directly Inhibits Fascin-1

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Running Title: miR-143/145 Cluster is a Tumor Suppressor in Chondrosarcoma

Key Words: chondrosarcoma, microRNAs, tumor suppressor, biomarker

Status: Under revision at *Cancer Research* (Submitted January 17th, 2019, revision experiments ongoing since February)

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Abstract

Purpose: Chondrosarcoma is the second most frequent bone sarcoma. Due to the inherent chemo- and radiotherapy resistance and absence of known therapeutic targets, clinical management is limited to surgical resection. Consequently, patients with advanced disease face a poor prognosis. Hence, elucidating regulatory networks governing chondrosarcoma pathogenesis is vital for development of effective therapeutic strategies.

Experimental Design: miRNA and mRNA next generation sequencing of different subtypes of human chondrogenic tumors in combination with *in silico* bioinformatics tools was performed with the aim to identify key molecular factors. Validation of miRNA levels were further determined in a larger cohort of tumor and plasma samples from patients by qRT-PCR. The biological relevance of miR-143/miR-145 and their target fascin-1 (FSCN1) was studied in *in vitro* functional assays using chondrosarcoma cell lines with transient/stable cluster overexpression or target gene knockdown. Additionally, *in vivo* effects of miR-143/miR-145 overexpression were investigated in an orthotopic xenograft mouse model. A tissue microarray containing chondrosarcoma patient samples was immunostained and analyzed for FSCN1 expression.

Results: We demonstrate that miR-143/145 cluster expression inversely correlates with tumor grade and plays a tumor suppressor role *in vitro* and *in vivo*. Circulating miR-145 is highlighted as a potential non-invasive diagnostic biomarker. Furthermore, we newly identify FSCN1 as a direct cluster target and its knockdown had similar effects as miR-143/145 overexpression *in vitro*. Lastly, FSCN1 is a malignancy promoting factor associated with aggressive chondrosarcoma progression.

Conclusions: Our findings underscore miR-143/145/FSCN1 as important players in chondrosarcoma and open new avenues for specific therapeutic intervention options.

Introduction

Chondrosarcoma is the second most frequently diagnosed bone sarcoma after osteosarcoma (1). It has a broad spectrum of histological appearances, however, conventional chondrosarcoma accounts for 90% of all cases. The conventional subtype together with other less common chondrosarcoma variants, such as dedifferentiated, mesenchymal and clear cell chondrosarcoma belongs to a heterogeneous group of cartilage-producing neoplasms known as chondrogenic tumors. The World Health Organization (WHO) recognizes three categories of chondrogenic tumors: benign, intermediate and malignant (1). An increasing amount of evidence supports that benign tumors (enchondromas and osteochondromas) progress towards conventional chondrosarcoma, which is echoed in a challenging radiological and histological distinction between them (2).

In contrast to benign tumors, intermediate chondrogenic tumors (grade I chondrosarcoma) are locally aggressive and, although rarely metastatic, they can develop towards malignant subtypes that often metastasize (grade II or III and possibly to dedifferentiated chondrosarcoma) (3). Chondrosarcoma clinical management is limited to surgical resection, as conventional chemotherapy and radiotherapy are largely ineffective (4). Therefore, patients with inoperable or pulmonary metastatic disease have a poor prognosis. Although recent studies focused on the elucidation of factors contributing to chondrosarcomagenesis and progression (5-7), the molecular mechanisms remain poorly understood (8). Therefore, there is an urgent need to gain a deeper insight into chondrosarcoma biology in order to develop better diagnostic/prognostic tools and new therapeutic strategies that will ultimately improve patients' survival.

MicroRNAs (miRNAs) are a family of small non-coding RNAs of approximately 20–22 nucleotides in length (9). They participate in a wide variety of biological processes including

cell proliferation, differentiation, migration, apoptosis and others through posttranscriptional regulation of gene expression. In cancer, miRNAs are frequently deregulated and act either as tumor suppressors or oncogenes depending on the mRNA targets that they control (10). Importantly, miRNA expression profiles can be useful to distinguish tumor subtypes, predict patient survival or treatment responses. Additionally, miRNAs have been found to be remarkably stable in biological fluids, and therefore, are regarded as attractive non-invasive biomarkers (11). Several studies have shown involvement of miRNAs in chondrosarcoma and have identified targets, e.g. SOX9 and VEGF, known to be deregulated in chondrogenic tumors (12, 13). Nevertheless, evidence supporting the functional and clinical relevance of miRNAs in chondrosarcoma is still limited. Furthermore, potential diagnostic and prognostic values of circulating miRNAs have not been evaluated in cartilaginous tumors so far.

In the current study, we performed next-generation miRNA sequencing on a panel of human chondrogenic tumors with the aim to identify novel miRNAs involved in chondrosarcoma pathogenesis. Our findings demonstrate that miR-143-3p and miR-145-5p, two miRNAs transcribed as part of the same precursor, inversely correlate with tumor grade and play a tumor suppressor role *in vitro* and *in vivo*. Importantly, we categorize circulating miR-145-5p as a potent diagnostic biomarker for chondrogenic tumor subtypes. Finally, an integrative transcriptomic and bioinformatics approach allowed us to elucidate miR-143-3p/miR-145-5p direct target fascin-1 (FSCN1). Moreover, through tissue microarray analysis and loss of function studies we provide evidence that FSCN1 contributes to chondrosarcoma malignancy. Altogether, our study uncovers novel regulatory factors in chondrosarcoma progression.

Materials and Methods

Tissue specimens and patients' characteristics

All human tissue samples were collected between 1997 and 2016 at Balgrist University Hospital and University Hospital Zurich (Zurich, Switzerland) in accordance with the regulations of the local ethic committee (KEK ZH 2013-0430 and EK10/2007). The characteristics of patients with chondrogenic tumors and healthy individuals are summarized in Supplementary Tables S1, S2 and S3. For mRNA tumor tissue analysis, 42 specimens were collected at surgical resection, snap frozen in liquid nitrogen and stored at -80°C. Plasma samples from 42 patients were generated from peripheral blood draws 24 hours before and after surgery and kept at -80°C. The training and validation cohorts included 25 and 17 samples, respectively. Control blood was collected from 20 healthy individuals. A tissue microarray (TMA) block was prepared using tissue samples from 55 chondrosarcoma patients.

Cell culture

The human chondrosarcoma cell lines JJ012 and 105KC were kindly provided by S.P. Scully (University of Miami Miller School of Medicine, Miami, FL, USA) and SW1353 (HTB-94) cells were obtained from ATCC. Cells were cultured in DMEM (4.5 g/l glucose)/HamF12 (1:1) medium (61965026 and 1765029, ThermoFischer) supplemented with 10% heat inactivated fetal bovine serum (FBS, 10500, ThermoFischer) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The cells were mycoplasma-free. For miRNA induction experiments cell culture medium was supplemented with the indicated concentration of doxycycline (D9891, Sigma-Aldrich). The cell lines were authenticated by short tandem repeat DNA profiling (Microsynth) with a PowerPlex®16HS system (DC2101, Promega) and by comparison with the German Collection of Microorganisms and Cell Cultures database. Primary cell lines were generated from resections of benign chondrogenic tumors collected at Balgrist University Hospital and cultured as described in (14). Three randomly-selected cell

lines were used in our study: one enchondroma-derived (#4) and two osteochondroma-derived (#5 and #10).

Plasmid constructs, transient transfection and lentiviral transduction

In order visualize tumor cells within mouse tissues, chondrosarcoma cells were stably transduced with a LacZ gene and selected as described (15). Transient transfection was performed according to the manufacturer's instructions using Lipofectamine RNAiMax (13778030, ThermoFischer) and the following miRNA mimics: miR-145-5p (HMI0224), miR-143-3p (HMI0221) and scrambled negative control (HMC0002), all purchased from Sigma-Aldrich, and used at a final concentration of 8 nM.

Tetracycline-responsive cells were obtained by lentiviral transduction with pLenti CMV rtTA3 Hygro (3rd generation lentiviral reverse tetracycline-controlled transactivator 3), and selected with medium supplemented with 400 µg/mL of hygromycin (400051, Merck Millipore) during one week. This construct was a gift from Eric Campeau (Addgene plasmid 26730). pLTR143-5 plasmid containing the miR-143/145 precursor DNA cloned in the 3′ UTR of RFP and the empty vector pLTR (EV) were a kind gift from Dr. Alfredo Pagliuca. Cells equipped with the LacZ and rTA3 gene were transduced with pLTR143-5 or EV as described (16) and RFP cells were flow sorted 72 hours after induction with 1 µg/ml of doxycycline. This last step was repeated for a second round of sorting.

Control cells and cells with stable shRNA-mediated FSCN1 knockdown (sh FSCN1) were generated through lentiviral infection with control scrambled shRNA (guide sequence: TCCGCAGGTATGCACGCGTG) or a shRNA targeting FSCN1 (guide sequence: TTCCAGTTTGAAAGGCAAGGG), purchased at GenomeRNAi. Transduced cells were selected with medium supplemented with 1 µg/mL puromycin (A1113803, Gibco). All

lentiviral particles were prepared with the Lenti-Pac™ HIV Expression Packaging Kit (LT001, GeneCopeia) following the manufacturer's instructions.

RNA isolation

Total RNA isolates (miRNA enriched) from cell lines were obtained with a miRNeasy Mini Kit (217004, Qiagen), accompanied by on-column DNase-treatment (79254, Qiagen) according to the manufacturer's protocol. RNA from freshly frozen human or murine tumor tissue samples was extracted with a miRNeasy Micro Kit (217084, Qiagen). Between 20 and 30 mg of frozen tumor tissues were homogenized with TissueLyser LT (Qiagen) and processed according to the manufacturer's protocol. RNA from plasma samples was isolated as described previously (17).

RNA sequencing

For small RNA expression analysis, libraries were prepared with a TruSeq Small RNA Library Preparation Kit (Illumina) from 100 ng of total RNA extracted from eight randomly-chosen tumor samples (3 benign, 2 intermediate, 3 malignant) with homogenous RNA integrity numbers. Paired end sequencing of 100-nucleotide sequences was performed on an Illumina HiSeq4000 as a service by the Functional Genomics Center Zurich (http://www.fgcz.ch/). For total RNA expression analysis, the library preparation was done in the same samples with a TruSeq Stranded Total RNA kit (Illumina) complemented with a rRNA depletion step (Ribo-Zero Gold rRNA Removal Kit, Illumina). To detect differentially expressed genes/miRNAs, we used the software package EdgeR. The differential expression was assessed using an exact test adapted for over-dispersed data. miRNAs with following parameters: $|\log_2 FC| \geq 1.5$, $P < 0.05$ and FDR < 0.3 were considered as differentially expressed. Genes showing altered expression ($|\log_2 FC| \geq 1$) with adjusted (Benjamini and

Hochberg method) $P < 0.01$ were considered differentially expressed. Unsupervised hierarchical clustering was done using the Ward2 method. The input genes for sample clustering were selected based on the read count standard deviations across the samples. The top variable 500 genes were chosen for the final sample clustering.

Reverse transcription and qRT-PCR

For miRNA expression analysis, 500 ng of miRNA were reverse transcribed from RNA extracts in miScript HiSpec Buffer with a miScript II RT Kit (218161, Qiagen) according to the manufacturer's instructions. For mRNA expression analysis, 1 µg of total RNA was transcribed to cDNA with a High-Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor (4374966, Applied Biosystems) according to the manufacturer. Quantitative PCR conditions and primers used are detailed in Supplementary Materials and Methods. Briefly, miRNAs in plasma were corrected to *C. elegans* miRNA-39 and normalized to miRNA-93 levels (determined by NormFinder algorithm (18)). miRNAs and mRNA levels in cell lines and tumor samples were normalized to the housekeeping RNU6B or GAPDH, respectively.

***In vitro* functional assays**

Short-term adhesion of cells to collagen I (C3867, Sigma) or fibronectin (F2006, Sigma Aldrich) and soft-agar colony formation assay were performed as detailed in Supplementary Materials and Methods. Cell cycle analysis and transwell migration assay were done as previously reported (19, 20).

Paratibial human xenograft chondrosarcoma mouse model

The *in vivo* study was conducted with approval of the Ethics Committee of the Canton Zurich Veterinary Office, and in accordance with the Swiss Animal Protection Law. Immunocompromised 6- to 7-week-old female SCID/CB17 mice were obtained from Charles

River Laboratories 14 days before tumor cell injection (TCI), done on day 0 of the experiment. For orthotopic TCIs, mice were paratibially injected with $4x10^5$ doxycycline-responsive JJ012/lacZ/EV (18 animals) or JJ012/lacZ/miR-143/145 (18 animals) cells in 50 µL of 1:1 PBS/0.05% EDTA and Matrigel (356234, Corning, 9.0 mg/ml protein concentration) as described (21). Primary tumor growth was detectable (by eye, caliper measurement or X-ray) four weeks after the TCI and mice were subsequently randomized according to their weight into the following four experimental groups: EV (n=7), EV+DOX (n=11), MIR (n=7) and MIR+DOX (n=11). Mice were once treated intraperitoneally with either sterile PBS (vehicle control) or doxycycline solution (53.3 mg/kg body weight). The mice were thereafter fed with doxycycline-supplemented food (625 mg doxycycline/kg) or control food (produced by KLIBA NAFAG) until day 61 after TCI, when they were euthanized as reported (22). Tumor development monitoring and processing of primary tumor and lung tissue was done as described previously (19). Indigo-blue stained pulmonary metastases on the lung surface were counted at 4x magnification under a Nikon Eclipse E600 microscope (Nikon Corporation).

Screening of miRNA targets

miR-143-3p and miR-145-p potential target genes were investigated with three different *in silico* prediction on-line tools: TargetScan (www.targetscan.org), miRDB (http://www.mirdb.org/) and MicroRNA.org (www.microrna.org/microrna). The list of the top 100 targets predicted by *in silico* tools was complemented with the mRNA expression data obtained by sequencing of patient primary tumor samples. Only genes predicted by the bioinformatic analysis and upregulated in malignant chondrosarcoma samples were considered for further validation. Finally, we tested for the expression levels of those selected genes in mimic-transfected miR-143/5 or control JJ012 and SW1353 cells by qRT-PCR.

Western blotting

Protein extract preparations and Western blot analysis were performed as previously described (19). Antibodies used are listed in Supplementary Materials and Methods.

Luciferase activity assay

To test the direct interaction between the studied miRNAs and FSCN1, we co-transfected a miRNA Target Expression Vector (E1330, Promega) containing the FSCN1 3'-UTR (either wild-type or mutated) together with miR-143, miR-145 or control mimics in JJ012 cells and we measured the Firefly and Renilla luciferase activities 48 hours later. Detailed protocol is reported in Supplementary Materials and Methods.

Human chondrosarcoma tissue microarray construction and analysis

Biopsies with histologically confirmed non-necrotic tumor tissue from surgical specimens obtained from 55 chondrosarcoma patients were marked, and two tissue cores per tumor and normal bone specimens with a diameter of 0.6 mm were arranged in a tissue array as described (23). A 2-µm section of the tissue array was transferred to an adhesive-coated slide system (Superfrost Plus, ThermoFisher Scientific, Waltham, MA USA), deparaffinized, and stained with an antibody against FSCN1 (mouse monoclonal antibody, dil. 1:400; MS-1112-S1, ThermoFischer), following standard procedures. The immunostaining analysis was performed as previously reported (19).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc, USA). Statistical significance of differences between the experimental groups was determined using a one or two-way ANOVA test with Bonferroni post-test. Student's t test

(unpaired) was used in miRNA-mimics and FSCN1 knockdown experiments. The correlation of miRNA levels was determined by Pearson correlation analysis. The experiments were performed in at least three independent replicates and results are shown as mean ± standard error of the mean (SEM). The receiver operating characteristic (ROC) curves were generated as described (17). The association between FSCN1 staining (positive/negative) and tumor grade in the TMA study was evaluated by the Chi-square test (Fischer's exact test). The results were considered significant when $P < 0.05$.

Results

Sequencing of small RNAs reveals miR-143/145 to be downregulated in chondrosarcoma

With the aim to identify novel regulators of chondrosarcoma pathogenesis, we analyzed both miRNA and mRNA expression profiles in primary tumor tissues collected from patients diagnosed with chondrogenic tumors of different malignancies. We applied next-generation sequencing to 3 benign chondrogenic tumors, 2 intermediate chondrosarcomas and 3 malignant chondrosarcomas. Unsupervised hierarchical clustering of mRNA expression profiles resulted in a better separation of tumor groups than the same analysis obtained from miRNA expression data (Supplementary Fig.S1). Nevertheless, we identified 43 down- (Fig. 1A and B) and 59 upregulated miRNAs in malignant versus benign tumors (Supplementary Fig. S1). In the current study, we focused on downregulated miRNAs that could be potential tumor suppressors.

Among the downregulated miRNAs, our attention was particularly drawn to miR-143-3p and miR-145-5p (subsequently referred to as miR-143/145) as the only broadly conserved full miRNA cluster/family present within the top hits. We focused on a miRNA cluster, as clusters can have a more complex target gene network than single miRNAs (24, 25). Noteworthy, both

miRNAs were among the most abundant small RNAs in the samples (Fig. 1C), representing at least 9.15% of all retrieved miRNA sequences and further implying their relevance for normal cartilage homeostasis.

In order to validate our finding, we performed miR-143/145 expression analysis in a broader panel of benign, intermediate and malignant chondrogenic tumors by quantitative RT-PCR. Consistent with our sequencing data, miR-143 and miR-145 expression was significantly decreased in malignant compared to benign specimens (Fig. 1D). The levels of the two miRNAs showed, as expected, a strong linear correlation (Fig. 1E) supporting the fact that both molecules are synthesized from the same precursor. We next evaluated miR-143/145 expression in primary benign tumor-derived cells and established human malignant chondrosarcoma cell lines (Fig. 1F). In agreement to what we observed in tumor samples, established chondrosarcoma cells lines expressed lower levels of miR-143/145 compared to primary cells derived from benign tumors.

In conclusion, miRNA profiling identified miR-143/145 cluster as a potential regulator of chondrosarcoma development.

Diagnostic value of plasma miR-143 and miR-145 in chondrosarcoma

Circulating miRNAs are widely investigated as cancer diagnostic markers in various cancer types (26). Thus, we next determined the levels of miR-143/145 in the plasma of healthy controls and patients with chondrogenic tumors by qRT-PCR (Fig. 2A). The results show a significant reduction in the circulating levels of both miRNAs in preoperative patient plasma compared to healthy controls, regardless the malignancy. Importantly, levels of miR-143 and miR-145 also displayed in the circulation a strong linear correlation (Fig. 2B). To assess the potential utility of plasma miR-143/145 levels as a non-invasive diagnostic marker for chondrosarcoma, we performed ROC curve analysis using training and validation cohorts as

well as their combination (Supplementary Fig. S2). As illustrated in Fig. 2C and D, an analysis designed to distinguish patients with malignant chondrogenic tumors from healthy controls yielded AUC values of 0.8813 (95% CI, 0.7601-1.002) and 1.000 (95% CI, 1.000-1.000) for miR-143 and mir-145, respectively. Additionally, miR-145 plasma levels proved to be useful to differentiate patients with malignant disease from patients with benign tumors with AUC value of 0.7578 (95% CI, 0.5869-0.9288; Fig. 2E). We next compared the miR-143/145 levels in the circulation of individual patients before and after tumor removal (Fig. 2F). Intriguingly, we found a statistically significant increase in the levels of both miRNAs in postoperative plasma.

Thus, our results imply that downregulation of miR143/145 cluster in the bloodstream is associated with chondrosarcoma progression. Furthermore, circulating miR-145 can be utilized to distinguish patients with different types of cartilaginous tumors.

miR-143/145 overexpression reduces the oncogenic properties of chondrosarcoma cell lines *in vitro*

In order to investigate the functional relevance of miR-143 and miR-145 for chondrosarcoma malignancy, both miRNAs were artificially upregulated in two established chondrosarcoma cell lines, JJ012 and SW1353, using transient or stable overexpression systems. For short-term experiments we used the transient transfection approach. Considering that constitutive overexpression of a potential tumor-suppressor might lead to complete growth arrest or induce counter-selection, we established an inducible lentiviral system of stable cell lines which conditionally overexpressed the miR-143/145 cluster in a doxycycline-dependent manner (16). Overexpression of both miRNAs in the two cell lines following transient transfection (Fig. 3A) or doxycycline induction was confirmed via quantitative RT-PCR (Fig. 3B). Transient transfection of miR-143/145 mimics in JJ012 cells resulted in

increased attachment to collagen ($P < 0.05$) and fibronectin ($P > 0.05$) (Fig. 3C), two extracellular matrix ligands highly abundant in the cartilage. There were no apparent differences in the proliferation and migration rates of both chondrosarcoma cell lines upon miR-143/145 overexpression (data not shown). However, a flow cytometric analysis of cell-cycle progression upon miR-143/145 cluster overexpression revealed a G1 cell-cycle arrest in JJ012 cells (Fig. 3D). We next studied the effects of long-term miR-143/145 induction on anchorage-independent growth using a soft-agar assay and found that both chondrosarcoma cell lines, upon induction of cluster upregulation, have a reduced ability to form colonies compared to control cells (Fig. 3E).

Collectively, the *in vitro* findings presented here implicate that miR-143/145 cluster overexpression reduces the malignant properties of the investigated chondrosarcoma cell lines by affecting adhesion, cell cycle progression and colony forming capacity.

miR-143/145 overexpression impairs tumor growth *in vivo*

Based on the observation that forced expression of the miR-143/145 cluster decreases the oncogenic features of chondrosarcoma cell lines *in vitro*, we subsequently explored whether the cluster overexpression has an impact on primary tumor progression and metastasis development *in vivo* using a xenograft orthotopic mouse model. To this end, JJ012/LacZ cells stably transduced with doxycycline-inducible expression vectors carrying either empty vector alone (EV) or miR-143/145 (MIR) were paratibially injected into immunocompromised SCID mice. Doxycycline or control treatment was started four weeks after tumor cell injection, namely as soon as the primary tumor formation was detectable. As expected, miR-143/145 induction caused a substantial inhibition of tumor growth, as reflected by significantly smaller relative tumor volumes in the MIR+DOX group of animals compared to the control EV, EV+DOX and MIR groups (Fig. 4A). Of note, primary tumor development, monitored by X-

Ray imaging over time, was associated with increasing osteolytic bone lesions in all the experimental groups (Fig. 4B). The efficacy of our doxycycline-inducible expression system was also confirmed *in vivo* by determining miRNA levels in tumors harvested from animals at the end of the experiment (Fig. 4C). In line with the *in vitro* findings, the induction of miR-145 was considerably more efficient than the miR-143 expression induction. It is, however, worth mentioning that a certain leakiness of the transgene expression is present in the absence of doxycycline.

Importantly, we found an inverse correlation between miRNA levels and tumor volume (Fig. 4D). We then investigated whether miR-143/145 cluster interferes with the metastatic dissemination of chondrosarcoma cells. In contrast to its potent tumor-suppressive activity, miR-143/145 cluster did not significantly impact the number of X-Gal stained pulmonary metastases (Fig. 4E and F). Noteworthy, we observed a tendency towards a decreased number of metastatic nodules in the lungs of animals orthotopically injected with JJ012/LacZ/miR-143/145 cells, regardless of doxycycline treatment, compared to animals bearing JJ012/LacZ/EV-derived tumors.

Taken together, our *in vivo* data clearly demonstrate that the miR-143/145 cluster inhibits primary tumor growth without affecting the metastatic spread of chondrosarcoma cells.

Identification of FSCN1 as a direct miR-143/145 cluster target in chondrosarcoma

To elucidate the underlying mechanisms of tumor-suppressive effects of miR-143/145 cluster, we developed a combinatorial analysis strategy to identify putative miR-143/145-regulated gene targets. The list of targets predicted by three *in silico* tools (TargetScan, miRDB and microrna.org) was complemented with the mRNA expression data obtained by sequencing of patient primary tumor samples. Taking into account that miRNAs primarily negatively regulate their target's mRNA and/or protein expression, we hypothesized that

levels of the miRNAs in clinical samples should inversely correlate with the levels of putative target genes. Therefore, only genes predicted by the bioinformatic analysis and upregulated in malignant chondrosarcoma samples were considered for further validation (Fig. 5A). The same workflow was applied for each prediction tool, and we shortlisted our candidates to only those genes identified as targets by all three databases (Fig. 5B). The normalized counts of the selected genes in tumor samples (4 for miR-143 and 4 for miR-145) are summarized in a heatmap (Fig. 5C). To experimentally validate our target screening approach, qRT-PCR expression analysis of the eight candidate genes in control cells and transiently overexpressing miR-143/145 JJ012 and SW1353 cell lines was performed (Fig. 5D). Among all tested genes, only FSCN1 was significantly downregulated in both cell lines in response to miR-143 and miR-145 mimics. Next, we analyzed FSCN1 protein expression in control and miR-143 or miR-145 overexpressing cells and found that FSCN1 levels were markedly reduced by both individual miRNAs in both cell lines (Fig. 5E). The same was true in cells with stable miR-143/145 overexpression (Supplementary Fig. S5). In line with these findings, FSCN1 protein expression inversely correlated with miR-143/145 levels in primary tumors of doxycycline-treated animals (Supplementary Fig. S5). Overall, we demonstrated that miR-143/145 negatively regulated FSCN1 expression both *in vitro* and *in vivo*. In order to validate FSCN1 as a direct target of miR-143, miR-145 or both, we constructed a luciferase reporter containing the 3'-UTR region of the FSCN1 gene. According to TargetScan the FSCN1 3'UTR contains one and four conserved miRNA binding sites for miR-143 and miR-145, respectively. The results show that miR-143 and miR-145 significantly inhibited the luciferase activity of wild-type reporter in JJ012 cells when compared with the negative control. Importantly, mutagenesis of all conserved seed sites within the FSCN1 3'-UTR region abolished the inhibitory effect of miR-143 and miR-145 on the luciferase activity (Fig. 5F).

Collectively, our screening strategy followed by luciferase assays identified and confirmed FSCN1 as a direct target of both miR-143 and miR-145 in chondrosarcoma.

FSCN1 contributes to the malignant properties of chondrosarcoma cells *in vitro* and is upregulated in more aggressive human chondrosarcoma tumor specimens

FSCN1, highly expressed in many carcinomas, was shown to contribute to their aggressiveness (27). To evaluate the function of FSCN1 in chondrosarcoma, we first investigated the phenotypic effects of its downregulation in chondrosarcoma cells. The efficacy of stable shRNA-mediated FSCN1 knockdown was confirmed on mRNA and protein levels in both JJ012 and SW1353 cell lines (Fig. 6A and B). FSCN1 silencing did not influence adhesion to different ECM ligands or cell proliferation rates in 2D cell culture (data not shown). Interestingly, cell cycle progression analysis revealed a similar effect as previously observed in miR-143/145 overexpressing cells (Fig. 6C). Furthermore, although the total number of colonies grown in soft agar was not affected, both JJ012 and SW1353 cells with FSCN1 knockdown formed significantly smaller colonies in size compared to control cells (Fig. 6D). Finally, in agreement with the described role of FSCN1 in carcinomas, the migratory capacity of chondrosarcoma cells was significantly inhibited upon FSCN1 silencing (Fig. 6E). Considering the negative correlation between miR-143/145 and FSCN1 expression as well as the contribution of FSCN1 to the malignant phenotype of chondrosarcoma cells *in vitro*, we postulated that FSCN1 expression could be linked with the clinicopathological features of human chondrosarcoma. To test our hypothesis, we performed immunohistochemical staining for FSCN1 protein expression on a tissue microarray sections containing tumor specimens isolated from 55 patients diagnosed with Grade I, Grade II or Grade III chondrosarcoma (Fig. 6F). Indeed, the proportion of FSCN1 positively stained

samples in grade III specimens (100%) was significantly higher than in grade I (39%) and grade II (48%) ($P < 0.05$).

In summary, these results indicate that FSCN1 plays a tumor-promoting role and is associated with aggressive chondrosarcoma progression.

Discussion

The absence of efficient treatment options for chondrosarcoma patients with nonresectable or metastatic disease highlights the urgency for the discovery of novel therapeutic targets. Unravelling the mechanisms that govern tumor initiation and progression is therefore crucial in order to develop successful disease management and improve patient survival. With the intention to elucidate novel players in chondrosarcoma progression, we aimed at the identification of aberrantly expressed miRNAs by performing the Next-gen miRNA sequencing study in a panel of human chondrogenic tumors. In the past years, multiple individual deregulated miRNAs have been implicated in chondrosarcoma progression (28-30). Our data confirmed prior reports of dysregulation of several miRNAs (such as miR-200b, miR-125, miR-335, miR-183, miR-138, miR-222) (12, 31). In contrast to previously reported studies, our work focused on families or clusters of miRNAs, groups of commonly co-transcribed miRNAs that can jointly exert a more robust target gene regulation than single miRNAs (24, 25). We identified miR-143/145 as the only conserved downregulated miRNA cluster in chondrosarcoma that inversely correlated with tumor aggressiveness. Reduced levels of miR-143 or miR-145 have been reported in other tumor entities, and both molecules are generally acknowledged as tumor suppressors (32, 33). Our results are consistent with the findings recently published showing miR-145 to be downregulated in chondrosarcoma (34), however, the authors analyzed only a small cohort consisting of five clinical samples without

investigating the functionality of the miRNA. Importantly and in contrast to previous reports, our results are strengthened by an analysis of a considerably larger (42 samples) cohort. Furthermore, we provide a foundational piece of evidence on the use of circulating miRNAs as non-invasive biomarkers in chondrosarcoma, a valuable diagnostic tool in other sarcoma types (17). Both miRNAs were significantly downregulated in preoperative plasma of patients compared to healthy individuals. Strikingly, miR-145 plasma levels were lower in chondrosarcoma patients than in subjects with benign chondrogenic neoplasms (data confirmed in a validation cohort), suggesting that lower miR-145 is associated with a more aggressive disease, as seen for other miRNAs (35, 36). Given the observed power of circulating miR-145 alone to discriminate different chondrogenic tumor subtypes, we suggest that it can be useful as an non-invasive biomarker to improve chondrosarcoma diagnosis, which is a very challenging task (2). We believe that miR-145 in combination with additional deregulated miRNAs or other clinical features, including grading based on imaging (37), could facilitate an accurate assessment of tumor type, and potentially be translated into appropriate clinical management.

The fact that we observed miR-143/145 levels restoration upon tumor removal suggests that chondrosarcoma cells could regulate miR-143/145 secretion in neighboring cells (38). However, considering the short time period of sample collection after surgery, we cannot exclude that this effect was caused by other tissues possibly reacting to the surgical intervention, such as vascular smooth muscle cells (39). Therefore, later time points after resection as well as multicenter clinical validation in a novel cohort should be envisaged.

Subsequently, we sought to delineate the role of miR-143/145 cluster in chondrosarcoma cell biology by performing *in vitro* and *in vivo* functional studies upon miRNA expression manipulation. Our *in vivo* study employing an orthotopic xenograft model is the first

demonstration of the tumor-suppressive role of miR-143/145 in chondrosarcoma. Our findings reflect much of the literature regarding miR-143/145 roles in different cancer types (16, 40). Furthermore, the inhibition of primary tumor growth observed *in vivo* is in line with our *in vitro* data, where we show that miRNA enforced upregulation led to altered distribution of cell cycle phases and negatively affected the ability of cells to grow in an anchorage-independent manner. Cluster overexpression also increased the affinity of cells for extracellular matrix components abundant in the human cartilage, which could provide an advantageous ability of low-expressing miR-143/145 cells to escape the primary tumor site. On the other hand, we were not able to draw major conclusions regarding the association of miR-143/145 with metastasis formation, which has been previously reported in other tumor models (41) and would be of additional clinical relevance given the known burden caused by metastasis in chondrosarcoma patients (42). Our observations suggest that miR-143/145 do not affect the initial steps of metastasis. However, we could not assess the effects on the colonization and outgrowth in the lungs because, in our setting, most of the lesions were only visible under the microscope.

Another key finding of our work is the identification of FSCN1 as a direct target of miR-143/145 cluster in chondrosarcoma. FSCN1 is a gene that encodes an actin-bundling protein involved in the formation and functioning of cell protrusions relevant for cell motility and invasion (43). Evidence obtained from numerous cancer types has highlighted that miR-143 and miR-145 target FSCN1 (32, 44). In the current study, we found that miR-143 and miR-145 function as negative regulators of FSCN1, as we observed reduced FSCN1 expression following miR-143/145 upregulation and the direct interaction was confirmed in a luciferase assay. FSCN1 expression is frequently increased in carcinomas and is associated with a high risk of mortality and metastasis (27), making it a good prognostic marker in some epithelial

cancers (45). In contrast, only few studies have addressed the role FSCN1 in sarcomas (46, 47). To our knowledge, the here presented work is the first study investigating FSCN1 in chondrosarcoma. Our sequencing data indicated that FSCN1 mRNA is abundant in tumor specimens (data not shown) and an immunostaining analysis of a chondrosarcoma TMA revealed that the number of samples positively stained for FSCN1 increases with tumor grade. Similarly to other cancer models (48), depletion of FSCN1 in chondrosarcoma cells reduced their migratory capacity. FSCN1 knockdown cells induced a G1-phase cell cycle arrest and decrease in colony size in soft agar. Considering the fact that FSCN1 silencing only partially phenocopied the effects of miR-143/145 overexpression, we cannot exclude the possibility that the cluster mediates its function through downregulation of additional targets in chondrosarcoma. Nevertheless, we highlight FSCN1 as a novel oncogenic factor and an attractive therapeutic target in chondrosarcoma. It would be of great interest to test the efficacy of small molecule inhibitors of FSCN1 in future studies (49, 50).

Altogether, our study revealed that integrative miRNA-mRNA analysis in combination with *in silico* tools is a powerful and a robust approach for the discovery of chondrosarcoma regulators. We demonstrated that miR-143/145 cluster serves as a tumor-suppressor in chondrosarcoma. Both miRNAs exert their antitumor functions, at least in part, through a coordinated regulation of FSCN1 that we subsequently recognized as a malignancy promoting factor in chondrosarcoma pathogenesis. Moreover, we newly identified circulating miR-145 as a promising non-invasive biomarker. In conclusion, our findings underscore miR-143/145/FSCN1 as important players in chondrosarcoma progression. Restoration of miR145/145 levels in tumors or direct FSCN1 targeting are attractive potential options for therapeutic intervention in chondrosarcoma.

Authors' Contributions

Conception and design: J. Urdinez, A. Boro, A.Gvozdenovic

Development of methodology: J. Urdinez, A. Boro, A.Gvozdenovic

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Urdinez, A. Boro, A. Mazumdar, MJE Arlt, B. Fuchs, A.Gvozdenovic

Analysis and interpretation of data (e.g. statistical analysis, biostatistics, computational analysis): J. Urdinez, A. Boro, A. Gvozdenovic

Writing, review, and/or revision of the manuscript: J. Urdinez, JG Snedeker, A. Gvozdenovic

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Fuchs, R. Muff, SM Botter, B. Bode-Lesniewska

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Figure legends

Figure 1. The miRNA cluster miR-143/145 is downregulated in chondrosarcoma patient specimens. **A,** Volcano plot depicting the differential expression of miRNAs in malignant compared to benign chondrogenic tumors. Gray dots represent significantly up or downregulated miRNAs meeting the thresholds of log2 fold change above 1.5 and under −1.5, $P < 0.05$ and FDR < 0.30. **B,** List of the top downregulated miRNAs in malignant chondrosarcoma specimens (selected miRNAs are shown in bold). **C**, Normalized expression levels of top 500 most expressed miRNAs in tumor samples; the dotted line represent the 1:1 ratio, selected miRNAs (marked in black) are downregulated (bellow the line) and highly abundant. **D,** Relative miR-143 (left) and miR-145 (right) expression in human benign (n=17), intermediate (n=7) and malignant (n=18) chondrogenic primary tumor samples. The data are normalized to RNU6B levels. **E**, Pearson correlation between miR-143 and miR-145 levels in tumor samples (ΔCT values shown). **F**, miR-143 and miR-145 expression levels in primary tumor-derived cells and indicated established human chondrosarcoma cells lines. Values in D represent the median ± SEM, and values in F the mean ± SEM of miRNA expression. *, $P < 0.05$; ***, $P < 0.001$.

Figure 2. Diagnostic performance of miR-143/145 levels in the plasma of patients bearing chondrogenic tumors. **A,** Expression levels of miR-143 (left) and miR-145 (right) in the plasma of healthy subjects (n=20) and patients with benign (n=16), intermediate (n=10) and malignant (n=16) tumors depicted in dot plots (grey represents training and black validation cohort). miR-93 and c-39 were used as normalization controls. **B**, Pearson correlation of miR-145 versus miR-143 levels in plasma from all patients and healthy controls (ΔCT values shown). **C,** ROC curve plotted to distinguish healthy subjects from patients with malignant

chondrogenic tumors using plasma miR-143 or miR-145 (**D**) expression levels. **E,** ROC curve plotted to differentiate patients with benign from patients with malignant chondrogenic tumors using plasma miR-145 expression levels. **F,** miR-143 (left) and miR-145 (right) levels in plasma from the individual patients taken before (Pre-op) and after (Post-op) tumor removal (benign, n=9; intermediate, n=5; malignant, n=9). *, $P < 0.05$; ***, $P < 0.001$.

Figure 3. The miR-143/145 cluster overexpression exerts tumor-suppressive effects in chondrosarcoma cells *in vitro.* **A,** Relative miR-143/145 expression in indicated chondrosarcoma cell lines 48 hours upon transient transfection with miRNA or non-specific scrambled mimics (control). Results are presented as fold change over the control. **B,** Relative miR-143/145 expression in stably-transduced JJ012 (left) and SW1313 (right) cells 48 hours after induction with doxycycline (DOX). Fold change of miRNAs expression was normalized to the levels in DOX-induced cells carrying only the empty vector (EV). **C,** Short-term adhesion (% of seeded cells) to collagen or fibronectin of JJ012 cells, transiently transfected with miR-143/miR-145 or non-specific scrambled control. **D,** Cell cycle distribution (% of total cell population) of JJ012 cell line, assessed by flow cytometric analysis of propidium iodide-stained cells, 48 hours after miR-143/miR-145 or non-specific scrambled mimics transient transfection. **E,** Representative images (left panel) and quantification of crystal violet-stained colonies grown in a soft-agar assays of miR-143/145 DOX-induced or control (EV) stably transduced chondrosarcoma cell lines (right panel) after 4 weeks in culture. All values represent the mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4. miR-143/145 overexpression inhibits tumor growth *in vivo*. **A,** Primary tumor growth over time monitored by caliper measurements of the tumor volume at indicated time points in SCID mice after paratibial injection of JJ012*/lacZ* cells containing stably integrated empty vector (EV) or miR-143/5 (MIR) doxycycline-inducible expression constructs. Following tumor detection, at day 28 after tumor cell injection (TCI), DOX or control treatment was introduced. EV (n=7) and MIR (n=7) groups of mice were fed with control food, whereas EV+DOX (n=11) and MIR+DOX (n=11) groups were fed with DOX-supplemented food. Values represent mean ± SEM fold change in tumor volume normalized to the tumor-bearing leg volume in individual mice measured on the day of DOX treatment initiation. **B,** Representative X-Ray images of tumor bearing legs in indicated groups of animals taken at specified time points after tumor cell injection. **C,** miR-143/miR-145 expression levels in primary tumor tissue extracts from 25 randomly selected animals of designated experimental groups harvested at the end of the experiment. Values are normalized to mean miR-143/145 expression in the control (EV+DOX) group and expressed as mean ± SEM fold change. **D,** Pearson correlation between miR-143 and miR-145 levels (-ΔCT values shown) and the primary tumor volumes of 25 randomly selected mice. **E,** *ex vivo* quantification of X-Gal stained JJ012*/lacZ/-* derived pulmonary metastases. The data indicate the numbers of metastases on the whole lung surface of individual mice belonging to the indicated groups. **F,** Representative image of X-Gal stained JJ012*/lacZ/-* cell line-derived micrometastasis in a lung section observed under the microscope (4X magnification); arrows indicate the micrometastatic lesions. ns, no significance; *, $P < 0.05$; ***, $P < 0.001$.

Figure 5. Identification of novel miR-143 and miR-145 target genes in chondrosarcoma. **A,** Schematic illustration of the strategy for selecting candidate target genes of miR-143 and miR-

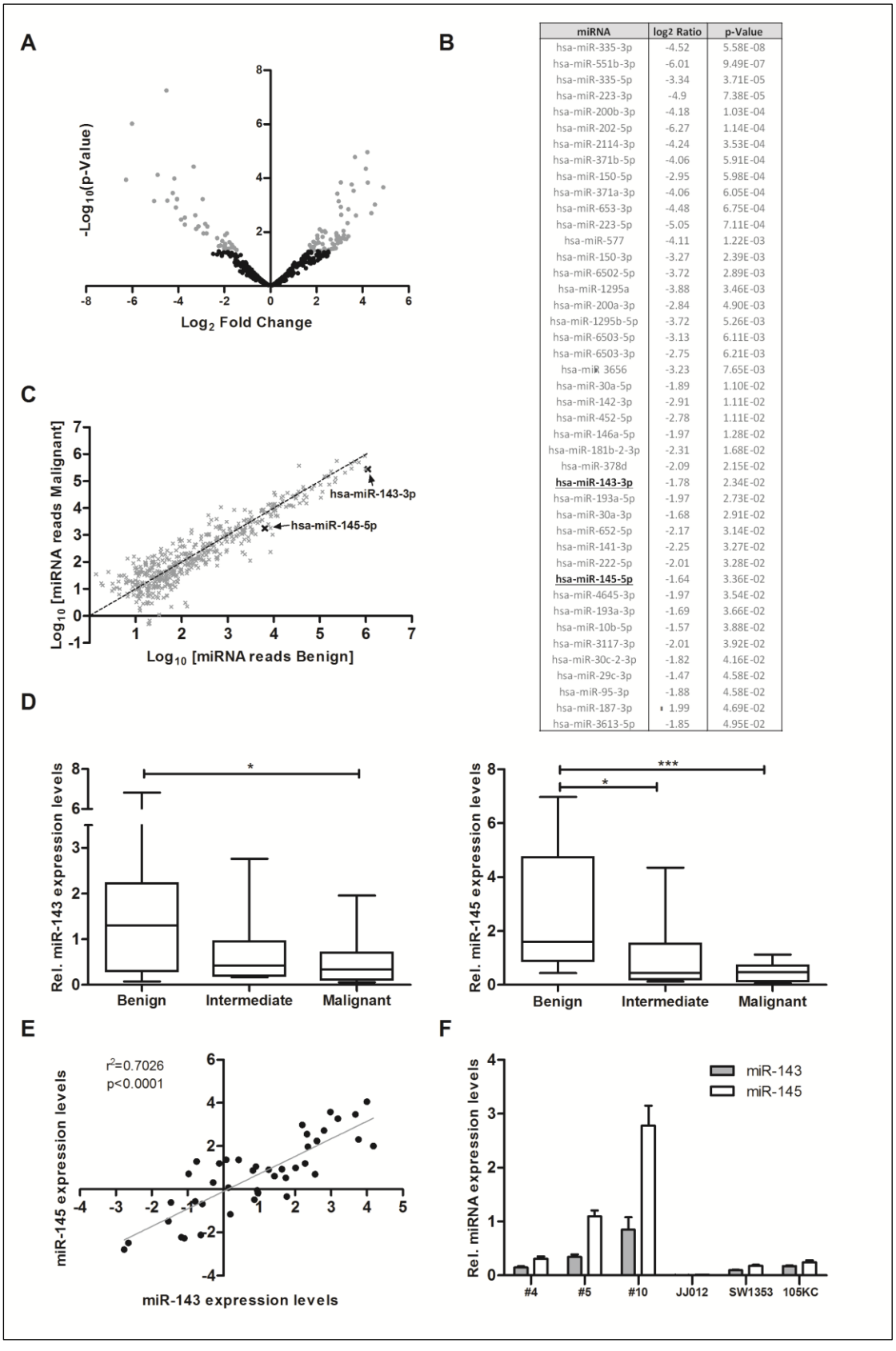
145 in chondrosarcoma **B,** Venn diagram displaying the number of miR-143 (left) and miR-145 (right) putative target genes, predicted by the indicated *in silico* platform tools. Numbers in black refer to unique targets of a particular platform, while numbers in white to targets predicted by two or three platforms. **C,** Heat-map depicting differential expression of the 8 selected miR-145 and miR-143 targets obtained by mRNA sequencing in tumor samples. Red color represents high relative expression levels and the blue color low expression. **D,** mRNA expression levels of putative miR-143/145 targets in JJ012 (left panel) and SW1353 cells (right panel) transiently transfected with miR-143/145 or non-specific scrambled control mimics determined by qRT-PCR. **E,** Western blot analysis of FSCN1 protein expression in whole cell extracts obtained from indicated cells transiently transfected with miR-143/145 or non-specific scrambled control (NC). Actin was used as a loading control. **F,** The human 3′ UTR of FSCN1 containing wild-type or mutated miR143/145 binding sites was cloned into the pmirGLO vector. Luciferase reporter constructs were transfected into JJ012 cells together with the indicated miRNA mimics and luciferase activity was measured after 48 hours. Firefly luciferase activity was normalized to Renilla luciferase activity. Values in D and F represent the mean ± SEM of three independent experiments. **, $P < 0.01$; ns, no significance.

Figure 6. FSCN1 contributes to *in vitro* oncogenic potential of chondrosarcoma cell lines and is associated with aggressiveness in human patient samples. **A,** FSCN1 mRNA expression in indicated chondrosarcoma cell lines upon stable shRNA-mediated knockdown (sh FSCN1). Expression levels are relative to GAPDH and normalized to negative control cells bearing scrambled shRNA (NC). **B,** Western blot analysis of total FSCN1 protein expression in whole cell extracts of the indicated chondrosarcoma cell lines. Actin was used as a loading control. **C,** Cell cycle was analyzed by flow cytometry. The graph represents cell cycle distribution (%

of total cell population) of specified chondrosarcoma cell lines. **D,** Representative images of crystal violet-stained colonies grown in soft-agar assays after 4 weeks in culture (left panel). The mean colony size (relative to control cells) of denoted cell lines (right panel). **E,** Transwell migration of indicated cells lines 24 hours after seeding. **F,** Representative images of chondrosarcoma tumor tissue microarray sections showing entire spots (upper panel) with nondetectable (left), moderate (middle), and intense (right) FSCN1 immunostaining. The proportion of negative (neg) and positive (pos) FSCN1 staining in chondrosarcoma clinical samples with different grading (lower panel). Values in A, C, D and E represent the mean ± SEM of at least three independent experiments. CS, chondrosarcoma; *, $P < 0.05$; ***, $P < 0.001$.

Figures

Figure 1



miRNA	log2 Ratio	p-Value
hsa-miR-335-3p	-4.52	5.58E-08
hsa-miR-551b-3p	-6.01	9.49E-07
hsa-miR-335-5p	-3.34	3.71E-05
hsa-miR-223-3p	-4.9	7.38E-05
hsa-miR-200b-3p	-4.18	1.03E-04
hsa-miR-202-5p	-6.27	1.14E-04
hsa-miR-2114-3p	-4.24	3.53E-04
hsa-miR-371b-5p	-4.06	5.91E-04
hsa-miR-150-5p	-2.95	5.98E-04
hsa-miR-371a-3p	-4.06	6.05E-04
hsa-miR-653-3p	-4.48	6.75E-04
hsa-miR-223-5p	-5.05	7.11E-04
hsa-miR-577	-4.11	1.22E-03
hsa-miR-150-3p	-3.27	2.39E-03
hsa-miR-6502-5p	-3.72	2.89E-03
hsa-miR-1295a	-3.88	3.46E-03
hsa-miR-200a-3p	-2.84	4.90E-03
hsa-miR-1295b-5p	-3.72	5.26E-03
hsa-miR-6503-5p	-3.13	6.11E-03
hsa-miR-6503-3p	-2.75	6.21E-03
hsa-miR 3656	-3.23	7.65E-03
hsa-miR-30a-5p	-1.89	1.10E-02
hsa-miR-142-3p	-2.91	1.11E-02
hsa-miR-452-5p	-2.78	1.11E-02
hsa-miR-146a-5p	-1.97	1.28E-02
hsa-miR-181b-2-3p	-2.31	1.68E-02
hsa-miR-378d	-2.09	2.15E-02
hsa-miR-143-3p	-1.78	2.34E-02
hsa-miR-193a-5p	-1.97	2.73E-02
hsa-miR-30a-3p	-1.68	2.91E-02
hsa-miR-652-5p	-2.17	3.14E-02
hsa-miR-141-3p	-2.25	3.27E-02
hsa-miR-222-5p	-2.01	3.28E-02
hsa-miR-145-5p	-1.64	3.36E-02
hsa-miR-4645-3p	-1.97	3.54E-02
hsa-miR-193a-3p	-1.69	3.66E-02
hsa-miR-10b-5p	-1.57	3.88E-02
hsa-miR-3117-3p	-2.01	3.92E-02
hsa-miR-30c-2-3p	-1.82	4.16E-02
hsa-miR-29c-3p	-1.47	4.58E-02
hsa-miR-95-3p	-1.88	4.58E-02
hsa-miR-187-3p	1.99	4.69E-02
hsa-miR-3613-5p	-1.85	4.95E-02

Figure 2

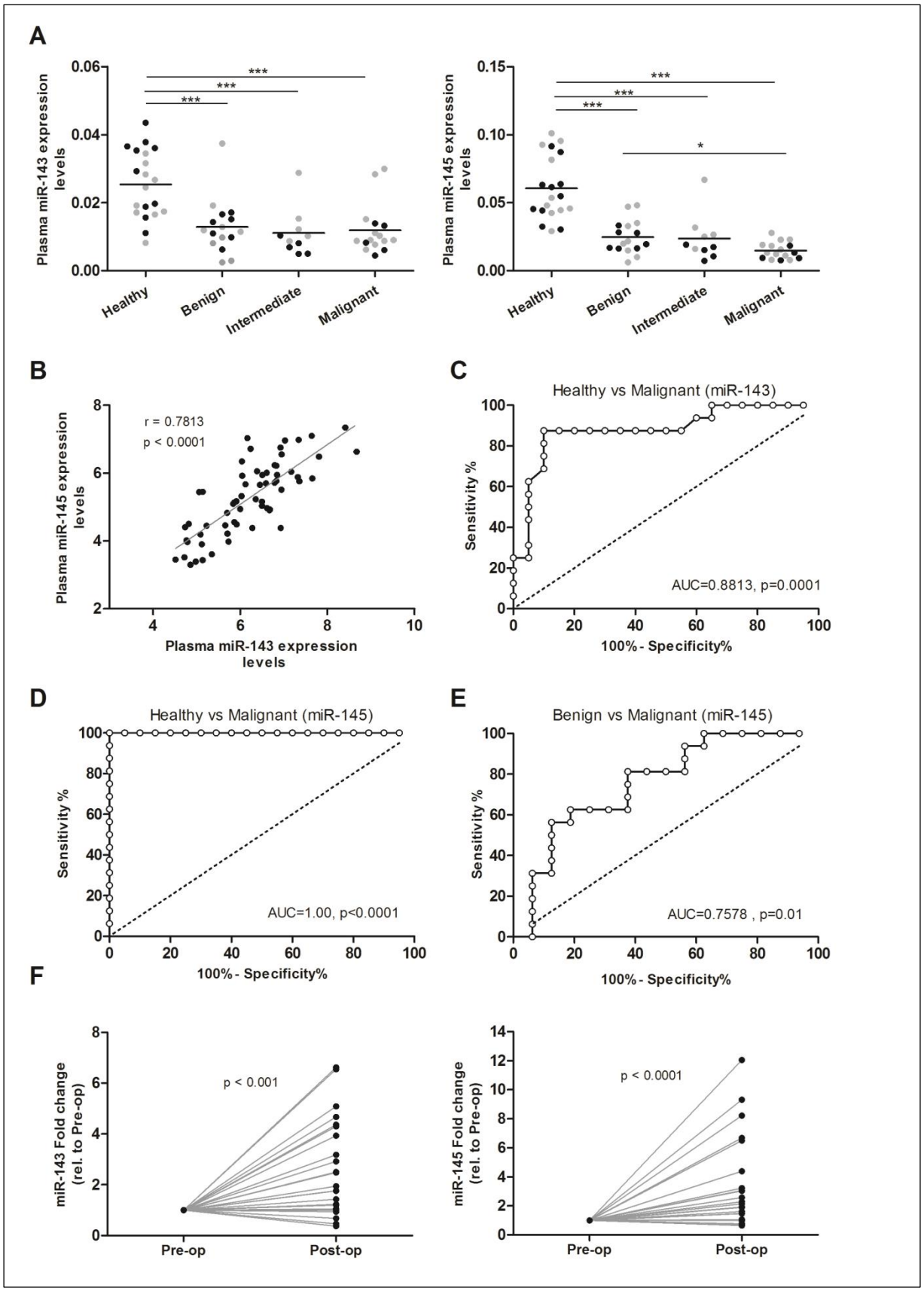

A
Plasma miR-143 expression levels
0.06
0.04
0.02
0.00
***
***
***
Healthy
Benign
Intermediate
Malignant
Plasma miR-145 expression levels
0.15
0.10
0.05
0.00
***
***
***
*
Healthy
Benign
Intermediate
Malignant
B
Plasma miR-145 expression levels
r = 0.7813
p < 0.0001
Plasma miR-143 expression levels
C
Healthy vs Malignant (miR-143)
Sensitivity %
AUC=0.8813, p=0.0001
100% - Specificity%
D
Healthy vs Malignant (miR-145)
Sensitivity %
AUC=1.00, p<0.0001
100% - Specificity%
E
Benign vs Malignant (miR-145)
Sensitivity %
AUC=0.7578 , p=0.01
100% - Specificity%
F
miR-143 Fold change (rel. to Pre-op)
p < 0.001
Pre-op
Post-op
miR-145 Fold change (rel. to Pre-op)
p < 0.0001
Pre-op
Post-op

Figure 3

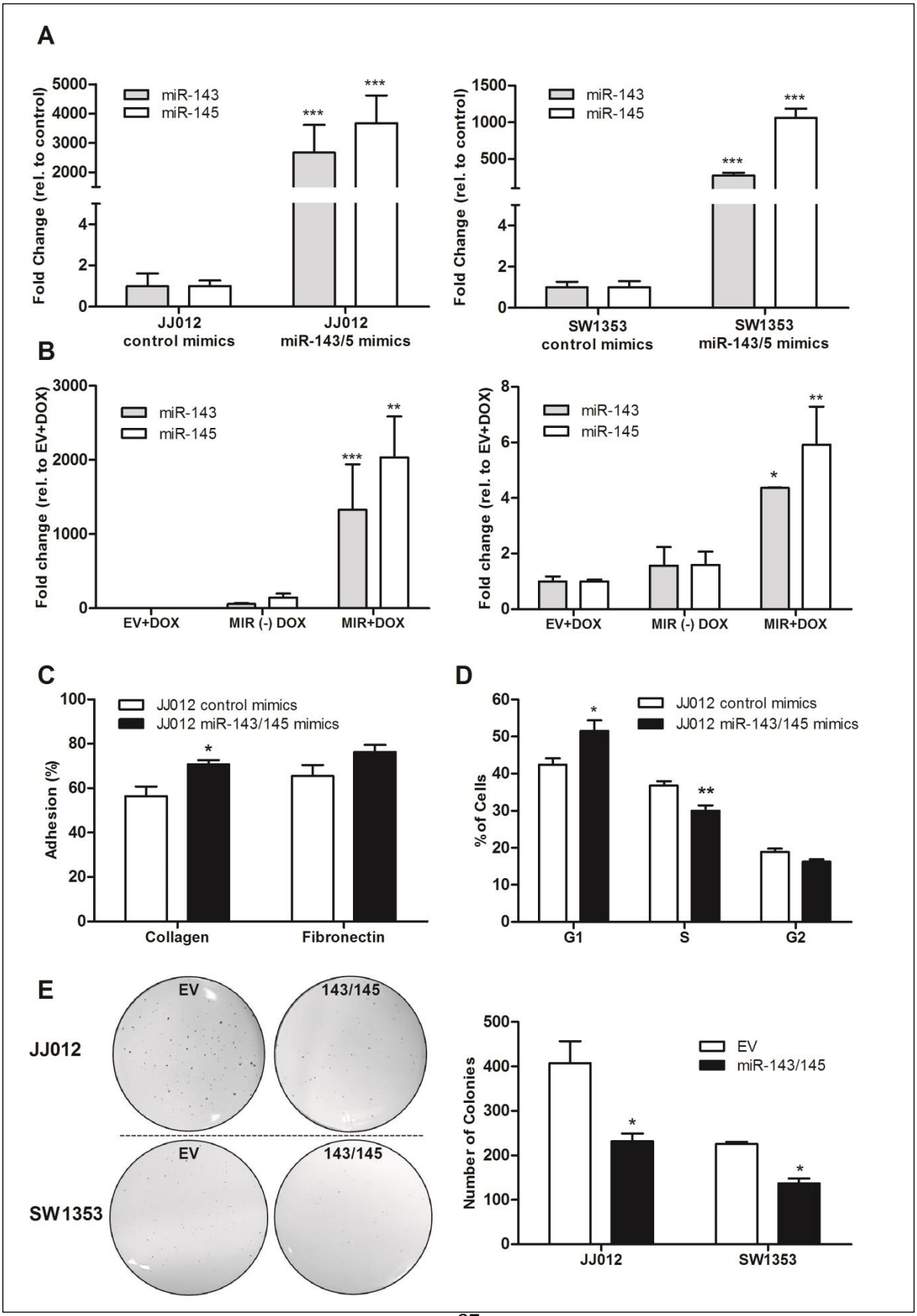

A
Fold Change (rel. to control)
miR-143
miR-145
JJ012 control mimics
JJ012 miR-143/5 mimics
SW1353 control mimics
SW1353 miR-143/5 mimics
B
Fold change (rel. to EV+DOX)
EV+DOX
MIR (-) DOX
MIR+DOX
C
JJ012 control mimics
JJ012 miR-143/145 mimics
Adhesion (%)
Collagen
Fibronectin
D
% of Cells
G1
S
G2
E
EV
143/145
JJ012
SW1353
Number of Colonies

Figure 4

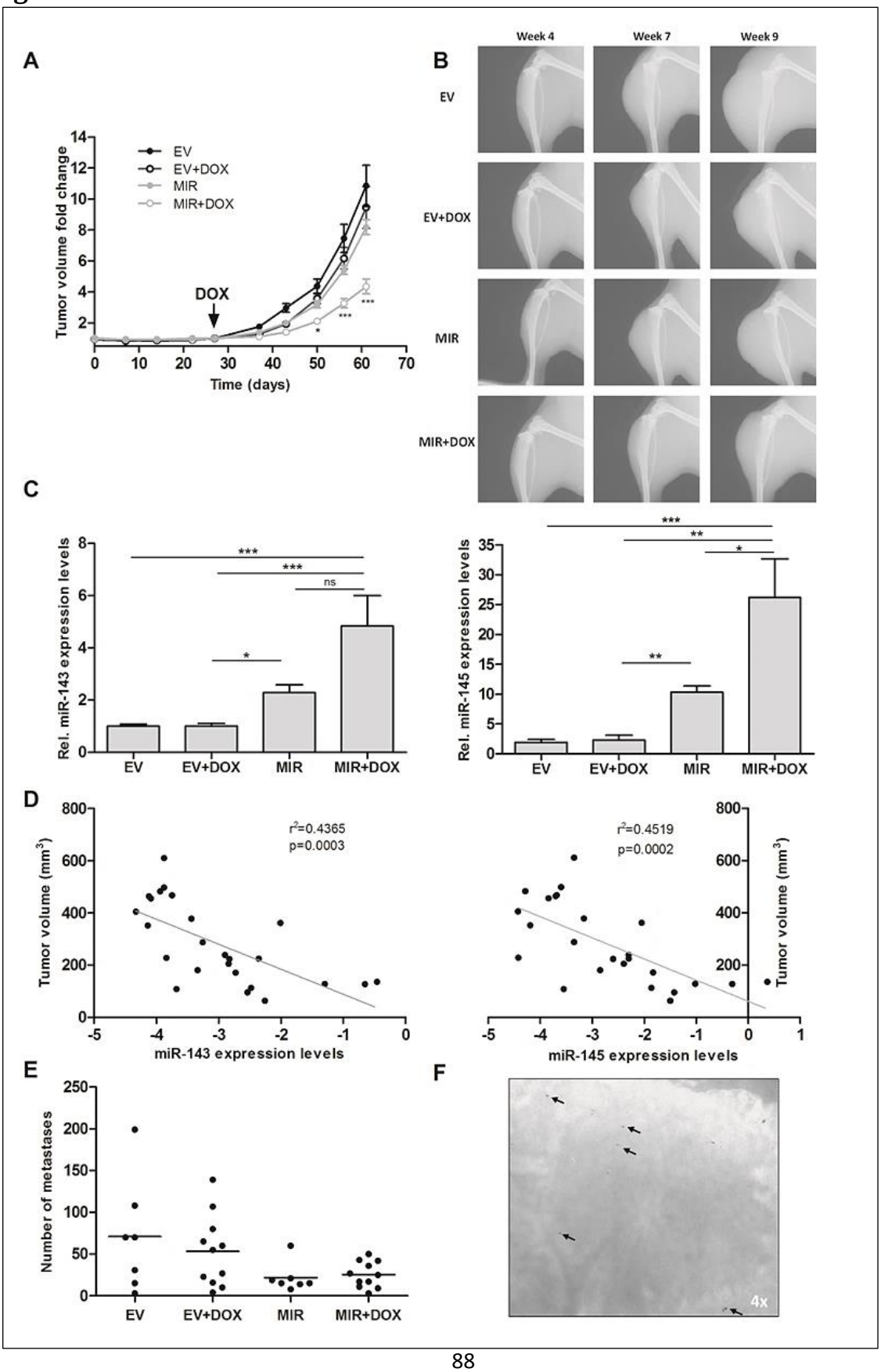

A
Tumor volume fold change
EV
EV+DOX
MIR
MIR+DOX
DOX
Time (days)
B
Week 4
Week 7
Week 9
EV
EV+DOX
MIR
MIR+DOX
C
Rel. miR-143 expression levels
Rel. miR-145 expression levels
EV
EV+DOX
MIR
MIR+DOX
D
Tumor volume (mm3)
r2=0.4365
p=0.0003
miR-143 expression levels
r2=0.4519
p=0.0002
miR-145 expression levels
E
Number of metastases
F
4x

Figure 5

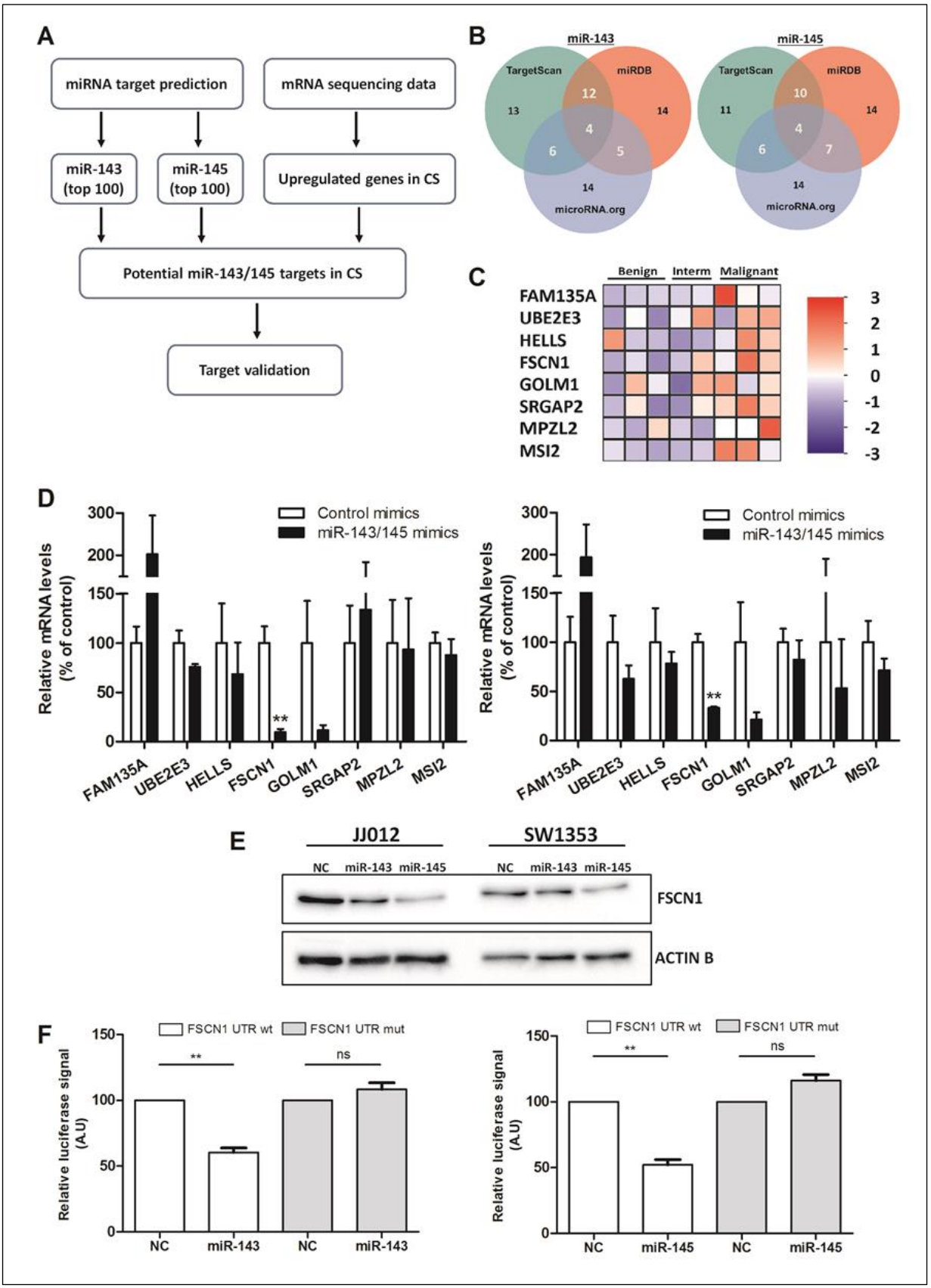
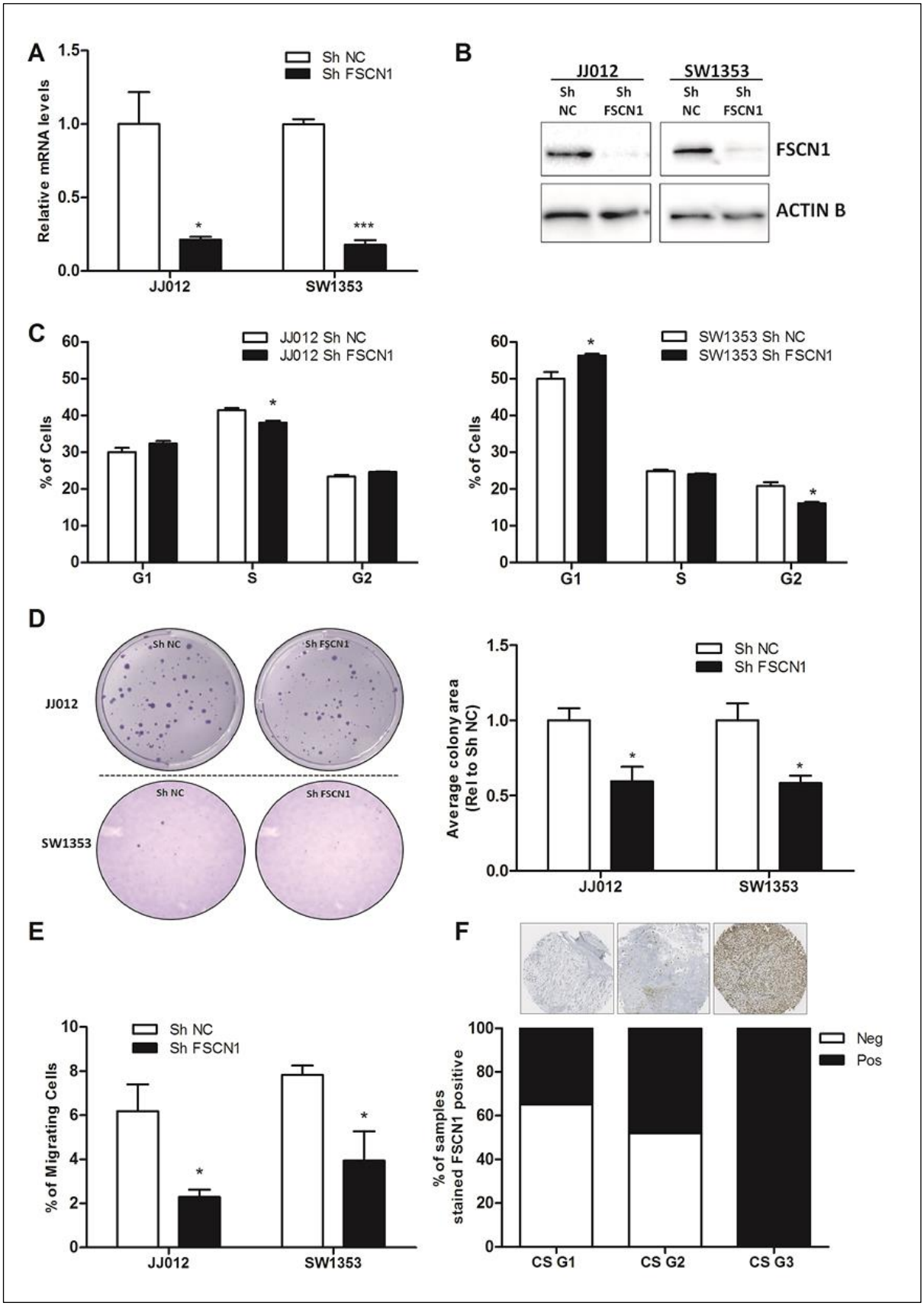

A
miRNA target prediction
mRNA sequencing data
miR-143 (top 100)
miR-145 (top 100)
Upregulated genes in CS
Potential miR-143/145 targets in CS
Target validation
B
miR-143
TargetScan
miRDB
microRNA.org
12
13
14
4
6
5
14
miR-145
TargetScan
miRDB
microRNA.org
10
11
14
4
6
7
14
C
Benign
Interm
Malignant
FAM135A
UBE2E3
HELLS
FSCN1
GOLM1
SRGAP2
MPZL2
MSI2
3
2
1
0
-1
-2
-3
D
Control mimics
miR-143/145 mimics
Relative mRNA levels (% of control)
300
200
150
100
50
0
**
FAM135A
UBE2E3
HELLS
FSCN1
GOLM1
SRGAP2
MPZL2
MSI2
Control mimics
miR-143/145 mimics
Relative mRNA levels (% of control)
**
E
JJ012
SW1353
NC
miR-143
miR-145
NC
miR-143
miR-145
FSCN1
ACTIN B
F
FSCN1 UTR wt
FSCN1 UTR mut
Relative luciferase signal (A.U)
150
100
50
0
**
ns
NC
miR-143
NC
miR-143
FSCN1 UTR wt
FSCN1 UTR mut
Relative luciferase signal (A.U)
**
ns
NC
miR-145
NC
miR-145

Figure 6


A
Relative mRNA levels
Sh NC
Sh FSCN1
JJ012
SW1353
B
JJ012
SW1353
Sh NC
Sh FSCN1
FSCN1
ACTIN B
C
% of Cells
JJ012 Sh NC
JJ012 Sh FSCN1
SW1353 Sh NC
SW1353 Sh FSCN1
G1
S
G2
D
Sh NC
Sh FSCN1
JJ012
SW1353
Average colony area (Rel to Sh NC)
E
% of Migrating Cells
F
% of samples stained FSCN1 positive
Neg
Pos
CS G1
CS G2
CS G3

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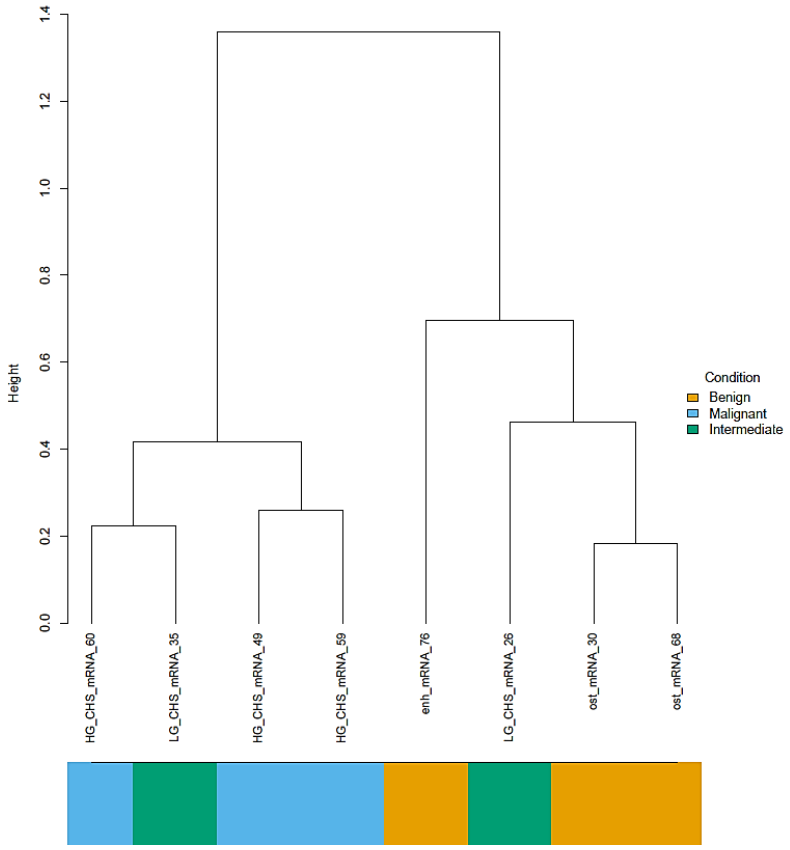
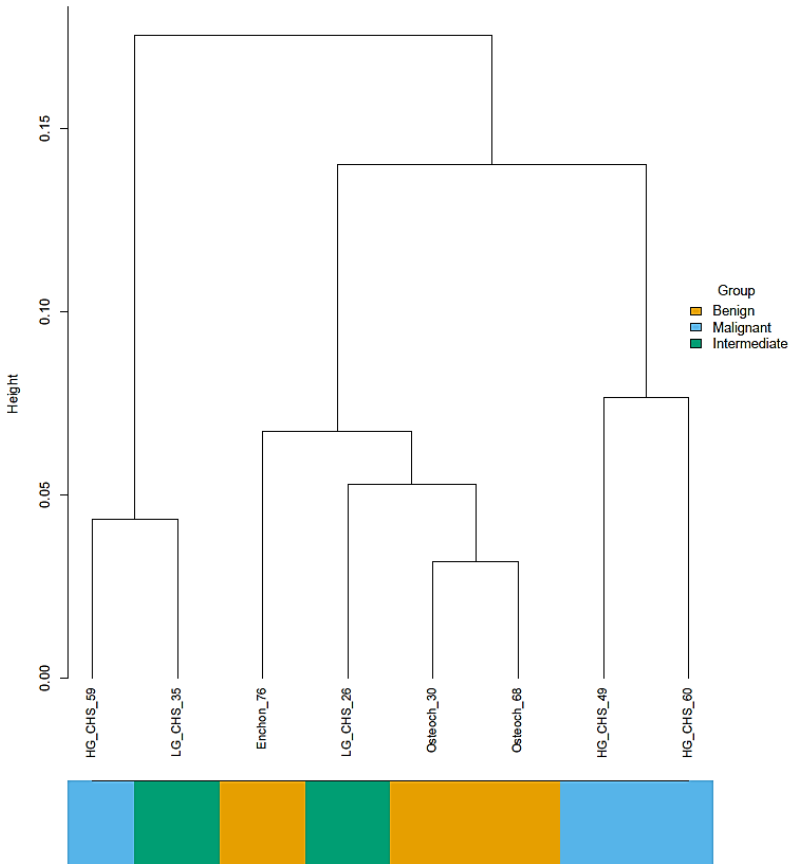
Supplementary Figure S1.

A



B



C

miRNA	log2 Ratio	pValue
hsa-miR-455-5p	4.21	0.0000109
hsa-miR-140-3p	3.66	0.0000165
hsa-miR-455-3p	4.14	0.0000445
hsa-miR-210-3p	3.05	0.000144
hsa-miR-346	4.22	0.000145
hsa-miR-138-5p	3.52	0.000172
hsa-miR-124-3p	4.9	0.000218
hsa-miR-675-3p	3.61	0.000291
hsa-miR-140-5p	2.9	0.000369
hsa-miR-149-5p	2.96	0.000718
hsa-miR-935	4.53	0.000948
hsa-miR-210-5p	3.05	0.00115
hsa-miR-26a-1-3p	3.38	0.00141
hsa-miR-433-3p	4.38	0.00198
hsa-miR-483-3p	3.07	0.00228
hsa-miR-138-1-3p	3.71	0.00242
hsa-miR-410-3p	3.34	0.00464
hsa-miR-574-3p	2.13	0.00791
hsa-miR-379-3p	3.17	0.00892
hsa-miR-203a-3p	2.36	0.00917
hsa-miR-6511b-3p	2.23	0.0093
hsa-miR-203b-5p	2.36	0.00936
hsa-miR-450a-2-3p	2.39	0.0101
hsa-miR-375	2.86	0.0109
hsa-miR-487b-3p	3.19	0.0128
hsa-miR-887-3p	2.03	0.0133
hsa-miR-377-5p	2.88	0.0136
hsa-miR-493-5p	2.93	0.0142
hsa-miR-431-5p	3.38	0.0143
hsa-miR-92b-5p	2.26	0.0151

hsa-miR-548h-5p	3.1	0.0167
hsa-miR-323a-3p	3.01	0.0168
hsa-miR-877-5p	2.04	0.0172
hsa-miR-654-5p	3.21	0.0175
hsa-miR-432-5p	3.29	0.0181
hsa-miR-323b-3p	2.61	0.0185
hsa-miR-329-3p	2.96	0.0189
hsa-miR-495-3p	2.87	0.0193
hsa-miR-320b	1.87	0.0227
hsa-miR-485-3p	3.05	0.0236
hsa-miR-103a-2-5p	2.09	0.024
hsa-miR-1180-3p	1.94	0.0255
hsa-miR-411-3p	2.92	0.0258
hsa-miR-543	2.7	0.027
hsa-miR-6511a-3p	1.98	0.0271
hsa-miR-153-3p	2.26	0.0279
hsa-miR-493-3p	2.75	0.0295
hsa-miR-1296-5p	1.7	0.03
hsa-miR-183-5p	1.88	0.0346
hsa-miR-23b-3p	1.76	0.0351
hsa-miR-668-3p	2.83	0.0397
hsa-miR-134-5p	2.33	0.0407
hsa-miR-487a-3p	2.73	0.0412
hsa-miR-337-3p	2.66	0.0419
hsa-miR-3687	2.53	0.045
hsa-miR-6894-3p	1.89	0.047
hsa-miR-370-3p	2.41	0.0481
hsa-miR-127-3p	2.16	0.0491
hsa-miR-483-5p	1.76	0.0493

Supplementary Figure S1: Sample clustering based on mRNA/miRNA expression signatures and miRNA differentially expression analysis. A, Nonsupervised hierarchical

clustering analysis based on the mRNA or miRNA **(B)** expression profiles in the sequenced tumor samples (3 benign, 2 intermediate, 3 malignant). The input genes for sample clustering were selected based on the read count standard deviations across the samples. Top most variable 500 genes/miRNAs were chosen for the final sample clustering. **C,** List of miRNA significantly upregulated in malignant versus benign chondrogenic tumors.

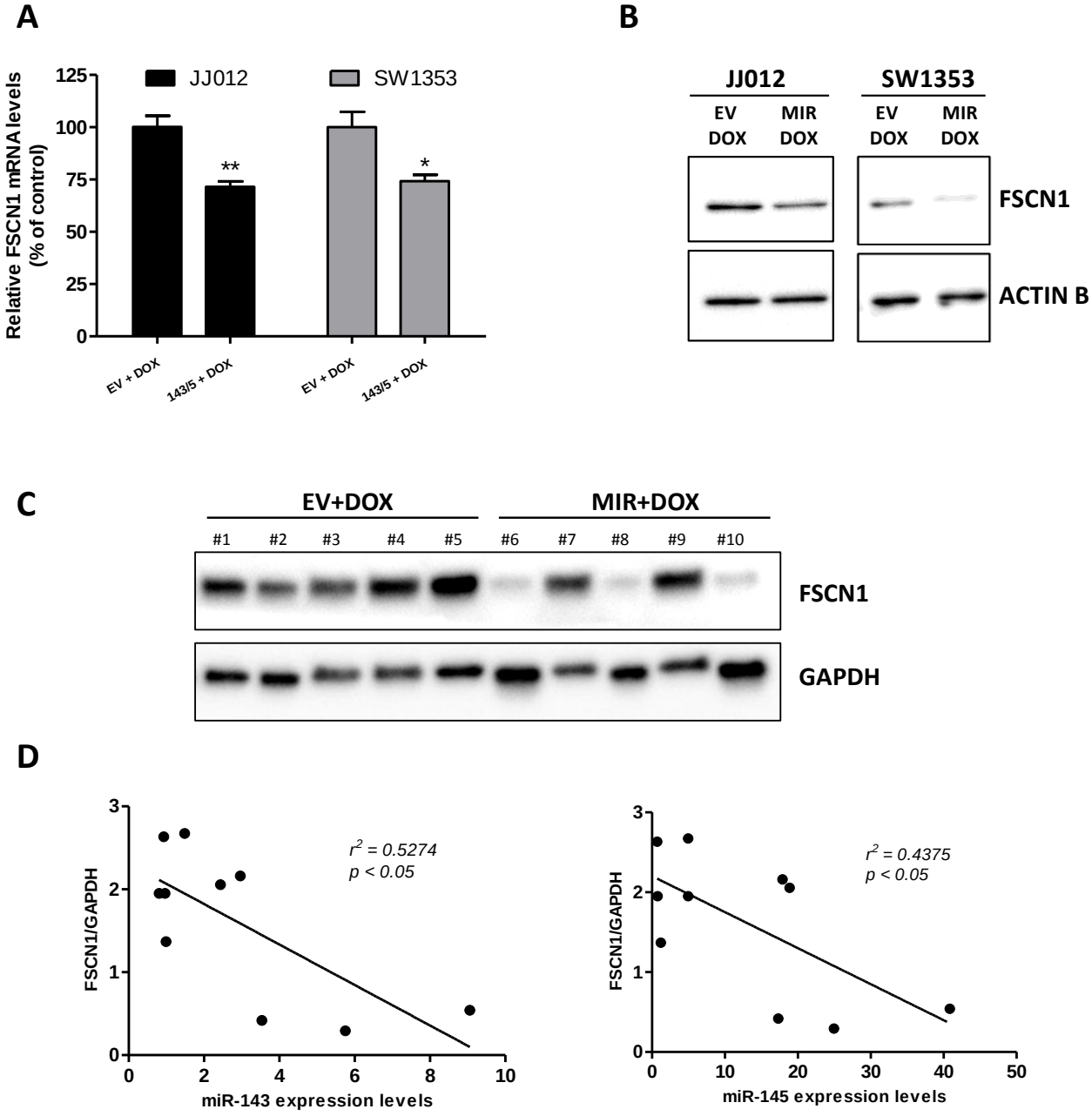
Supplementary Figure S2.

miR-145	Training		Validation		Combined	
Comparison	AUC	p-value	AUC	p-value	AUC	p-value
Healthy vs B	0.8833	*0.0048	0.9714	*0.00128	0.9203	*<0.0001
Healthy vs I	0.8600	*0.027	1.0000	*0.00221	0.9200	*0.00022
Healthy vs M	1.0000	*0.0001	1.0000	*0.00221	1.0000	*<0.0001
B vs I	0.6000	0.5485	0.8286	0.0618	0.5875	0.4606
B vs M	0.6768	0.1837	0.9143	*0.0185	0.7578	*0.0129
I vs M	0.8727	*0.02025	0.6400	0.4647	0.6813	0.1265

miR-143	Training		Validation		Combined	
Comparison	AUC	p-value	AUC	p-value	AUC	p-value
Healthy vs B	0.7889	*0.0338	0.9143	*0.00467	0.85	*0.00036
Healthy vs I	0.7600	0.1114	1.0000	*0.00221	0.905	*0.00036
Healthy vs M	0.8000	*0.0201	0.9600	*0.00486	0.8813	*0.0001
B vs I	0.6222	0.4634	0.8857	*0.0284	0.6125	0.3428
B vs M	0.5354	0.7903	0.7714	0.1229	0.5938	0.3658
I vs M	0.6545	0.3356	0.8000	0.1172	0.5438	0.7122

Supplementary Figure S2: ROC curve analysis of the training cohort, validation cohort and their combination for miR-145 (top) and miR-143 (bottom). B, benign; I, intermediate; M=malignant; *, $P < 0.05$.

Supplementary Figure S5.



Supplementary Figure S5: FSCN1 expression in miR-143/145 overexpressing cells *in vitro* and *in vivo*. A, Relative FSCN1 mRNA expression in stably-transduced chondrosarcoma cells 48 hours after induction with doxycycline (DOX). Fold change of mRNAs expression was normalized to the levels in DOX-induced cells carrying only the empty vector. **B,** Western blot analysis of FSCN1 protein expression in whole cell extracts obtained from

indicated cells induced with DOX for 48 hours. Actin was used as a loading control. **C,** Western blot analysis of FSCN1 protein expression in whole cell extracts obtained from primary tumor tissue of five different animals of each Dox-treated group. GAPDH was used as a loading control. **D,** Pearson correlation between miR-143 (left) or miR-145 (right) levels in murine primary tumor samples and relative amounts of FSCN1 (quantified by densitometric Western blot analysis and normalized to GAPDH).

Supplementary Table S1. Clinical characteristics of patients diagnosed with chondrogenic tumors (primary tumor tissue samples).

Variable		Benign	Intermediate	Malignant
Number		17	7	18
Age (years)				
	≤ 20	4	1	0
	20-40	8	3	4
	41-69	5	2	9
	≥70	0	1	5
Gender				
	Male	11	5	8
	Female	6	2	10
Tumor site				
	Femur	4	2	7
	Tibia	3	1	0
	Humerus	3	1	1
	Scapula	4	2	3
	Pelvis	1	0	6
	Other	2	1	1
Stage of disease				
	Localized	17	7	12
	Metastatic	0	0	6

Supplementary Table S2. Characteristics of healthy subjects and patients with chondrogenic tumors (plasma samples).

Variable		Healthy subjects	Discovery cohort	Validation cohort
Number		20	25	17
Age (years)				
	Mean	37.2	39.3	43.7
	SD	12.4	17.6	19.2
Gender				
	Male	13	12	13
	Female	7	13	4
Tumor type				
	Benign		9	7
	Intermediate		5	5
	Malignant		11	5
Tumor site				
	Femur		7	3
	Tibia		3	2
	Humerus		1	4
	Scapula		5	4
	Pelvis		4	3
	Other		5	1
Stage of disease				
	Localized		21	16
	Metastatic		4	1

Supplementary Table S3. Clinical characteristics of chondrosarcoma patients (TMA study).

Variable		Grade I	Grade II	Grade III
Number		23	25	7
Age (years)				
	≤ 20	5	0	0
	20-40	11	10	0
	41-69	6	10	6
	≥70	1	5	1
Gender				
	Male	15	13	4
	Female	8	12	3
Tumor site				
	Femur	4	8	0
	Tibia	1	0	0
	Humerus	3	1	0
	Scapula	1	2	3
	Pelvis	5	7	3
	Thorax	3	0	1
	Other	6	7	0
Stage of disease				
	Localized	23	21	5
	Metastatic	0	4	2

Supplemetary Table S4. List of primers utilized for Real-Time PCR.

Gene name	Fw Primer (5'-3')	Rev primer (5'-3')
FAM135A	TAG TGT AGA AGA AGA GGA TGG T	AAG ACG ATC AGT CAT GCT AT
UBE2E3	ACC ACT GCT AAG TTA TCC ACT A	ACA CCA CCT TCA TAT ACA GAA C
HELLS	CAT GTT TGG ATC CAG TGA GAA	TCC ACC TCT GGC TGT AT
FSCN1	GCAAGGACGAGCTCTTT	TTGGCAGACAGGTCCATAC
GOLM1	CCA ACC TGG AGA GGA AGT	CTG TCT CTG GTC GTT GTT T
SRGAP2	CAC CAA GGA CCA GCA AT	TCT TCC TGA GTC CTC GC
MPZL2	GCT CTA ACA GTG ACC TGG	TTC CCA TCC CAA GAC ACC C
MSI2	CAG AGG GTT TGG CTT TGT CA	CAT GGT GTA AGG CAG TCC
GAPDH	AAGGCTGGGGCTCATTTGCAGG	AGTTGGTGGTGCAGGAGGCA

Supplementary Table S5. List of primer used for site-directed mutagenesis in the specified miRNA seeding site at the FSCN1 3'-UTR.

Position in UTR (nt)	Fw Primer (5'-3')	Rev primer (5'-3')
miR-143 (1059-1065)	ACACTCTGGGTGTCCTGGTCTTTTATTTTTTG	CTAGCATGTCGGGGACGGGACG
#1 miR-145 (116-123)	ccaaACCCCAGAGAAAACGGTG	tcttTGAAAGGCAAGGGGGCTT
#2 miR-145 (377-384)	ccaaTCTCTTGCCCCTCCCAGC	tcttACACTACACGCCCAGGGC
#3 miR-145 (729-735)	CCTAGCCTGAgaccAAGCAGAAAATGAC	GTGAAAGGAAAGAGGAGG
#4 miR-145 (1140-1147)	ccaaAAATAGCGTCTAGAGTCG	tcttTTTGAAGCTACTATCATGG

Supplementary materials and methods

Reverse transcription and qRT-PCR

For miRNA expression analysis, 500 ng of miRNA were reverse transcribed from RNA extracts in miScript HiSpec Buffer with the miScript II RT Kit (218161, Qiagen) according to the manufacturer's instructions. After cDNA synthesis, the samples were diluted in 100 µL of water and 1 µL was then used as substrate for qPCR. Quantitative PCR was done using the miScript SYBR Green PCR Kit (218073, Qiagen) according to the manufacturer's instructions. The universal reverse primer provided in the kit was used in all reactions in combination with the following miScript Primer Assays (Qiagen) forward primers: Hs_RNU6-2_11 (MS00033740), Hs_miR-145_1 (MS00003528), Hs_miR-143_1(MS00003514), Hs_miR-128_1(MS00008582), Hs_miR-93_1(MS00003346), Hs_miR-214_2(MS00031605), Hs_miR-210_1(MS00003801), Hs_miR-16_2(MS00031493), Hs_miR-593*_1(MS00010283), Hs_miR-21*_1 (MS00009086), Hs_miR-141_1(MS00003507), Hs_miR-200a_1(MS00003738), Hs_miR-429_1(MS00004193). The PCR reactions were run on an Applied Biosystems Cycler under the following cycling conditions: initial denaturation at 95°C for 10 minutes, followed by 40 PCR cycles at 95°C for 15 seconds, 55°C for 30 seconds and 70° for 40 seconds. The levels of the investigated miRNAs in plasma samples were corrected for miRNA extraction efficiency, determined with the spiked-in C. elegans miRNA-39, and normalized to the levels of miRNA-93 that was determined with Norm Finder algorithm to be the least variable and therefore a suitable endogenous control (Fig. M1). In tissue samples and cell lines, RNU6-2 was considered as a housekeeping reference and used for normalization. Relative expression of miRNAs was calculated using the comparative (ΔΔCT) method.

A

Gene name	Stability value
miR-93	0.205
miR-16	0.207
miR-128	0.251
miR-429	0.259
miR-593	0.546
miR-210	0.211
miR-214	0.285

B

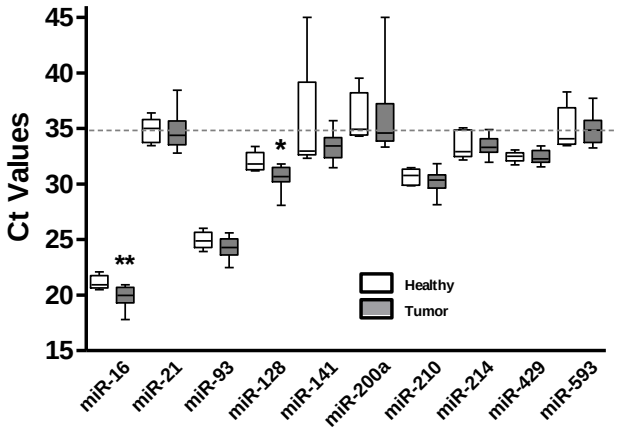


Figure M1. Selection of suitable housekeeping circulating miRNA. A, Stability analysis of tested miRNA in NormFinder. **B,** Ct values obtained by qRT-PCR of indicated miRNAs in randomly selected plasma samples from healthy samples (n=5) or chondrogenic tumor bearing patients (n=15). Boxes intersecting the dotted line and indicating low expression level were excluded from the analysis.

For mRNA expression analysis, 1 µg of total RNA was transcribed to cDNA with a High-Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor (4374966, Applied Biosystems) as described in the protocol provided by the manufacturer. The cDNA was diluted in nuclease-free water and real-time qPCR was conducted on cDNA equivalent to 10 ng of starting RNA with the Power SYBR Green PCR Master Mix (4367659, Applied Biosystems) on a StepOne-Plus Real-Time PCR System (Applied Biosystems). The denaturation was performed for 10 minutes at 95°C followed by 40 PCR cycles for 15 seconds at 95°C and for 1 minute at 60°C. The analysis was done with StepOne Software version 2.1 (Applied Biosystems). Relative expression levels were calculated by the comparative (ΔΔCT)

method and normalized to GAPDH. All primers used are listed in Supplementary Table S4. The experiments were done in triplicates and repeated three times.

Immunoblot antibodies

Antibodies used were anti-FSCN1 (sc-46675, dilution 1:1500) obtained from Santa Cruz Biotechnology, anti-β-actin (MAB1501, dilution 1:10000) from Merck Millipore, and anti-GAPDH (G9545, Sigma-Aldrich). HRP-conjugated secondary antibodies (sc-2054, sc-2055, dilution 1:3000) were purchased from Santa Cruz Biotechnologies.

Soft-agar colony formation assay

The experiments were carried out in 6-well cell culture plates containing 1.5 ml/well of 0.5% DNA grade agarose (V3121, Promega, base agar) in cell culture medium. Single cell suspensions (2x 10^4 or 1x10^4 cells per well for SW1353 or JJ012 derived cell lines, respectively) were prepared in 1.5 ml/well cell culture medium containing 0.35% agarose (top agar),seeded on the base agar and incubated in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Twenty-four hours later 1.5 ml/well of cell culture medium (supplemented with doxocycline 1µg/mL and PSA 1X) was added. Cells were cultured for 3 weeks and the medium was exchanged in 3-4 day intervals. Colonies were stained with 0.005% crystal violet. Images of entire wells or random fields (10x magnification) were taken with a Nikon Eclipse E600 microscope (Nikon Corporation) and the numbers of colonies were counted with the image processing program ImageJ (http://rsb.info.nih.gov/ij/). The results are presented as the mean ± SEM of three independent experiments.

Adhesion assay

Adhesion assays were carried out in 96-well plates. The wells were coated with 15 µg/ml per well of collagen I (C3867, Sigma) or fibronectin (F2006, Sigma Aldrich), yielding 5 µg/cm^2 of

substrate per well and incubated for 1 hour at room temperature (RT). After washing with PBS, the wells were blocked with heat-denatured (HD) 1% BSA in PBS for 1 hour at RT. Wells coated with HD-BSA alone were used as controls. Cells were detached with accutase (Sigma-Aldrich), resuspended in cell culture medium and 5 x 10^3 cells in 100 µl were seeded in triplicates into wells and allowed to adhere for 10 minutes at RT. Non-adherent cells were removed by washing with PBS and adherent cells were fixed with 10% formalin in PBS for 15 minutes at RT and then stained with 0.05% crystal violet in H2O for 15 minutes at RT. Images of randomly selected areas of 3.6 mm2 were taken with an AxioCam MRm camera connected to the Zeiss Observer.Z1 inverted microscope (Carl Zeiss MicroImaging GmbH) set at 4x magnification. The number of adherent cells in the analyzed area was determined with ImageJ software (http://rsb.info.nih.gov/ij/) and the total number of adherent cells per well was then calculated. The percentage of adherent cells presented in the graphs was calculated from the total number of adherent cells per well divided by the total number of seeded cells and multiplied by 100. The data of three independent experiments done in triplicates are presented.

Luciferase activity assay

The 3′UTR fragments of selected genes were amplified by PCR from JJ012 genomic DNA using high fidelity Pfu DNA Polymerase (M774A, Promega) according to manufacturer's protocol. The primers were the following: FSCN1 Fw: ATACTCGAGCAACCCTCCCTGCTAAC; FSCN1 Rev: GTCTCTAGACGCTATTTCCAGTTTGA. Of note, the primers contained XhoI and XbaI restriction sites for subsequent cloning strategy.

The PCR products were cloned into a pGEM®-T Easy Vector (Promega), and then subcloned into a pmirGLO Dual-Luciferase miRNA Target Expression Vector (E1330, Promega), downstream of the firefly luciferase open reading frame (ORF). Mutagenesis of the miR-143

and miR-145 seed motifs in the 3′ UTR of selected genes was performed using the Q5® Site-Directed Mutagenesis Kit (E0554S, New England Biolabs). Primer sequences are specified in Supplementary Table S5. Wild-type and mutated DNA sequences were verified by sequencing (Microsynth). DNA constructs were transfected to 5 x 103 HEK 293T or JJ012 cells in 96-well plates together with miRNA mimics using Lipofectamine 2000 (11668027, ThermoFischer) according to the manufacturer's instructions. Final amounts of DNA and miRNA mimics were 200 ng/well and 30 nM, respectively. Activity of Firefly and Renilla luciferase enzymes was measured using Dual-Luciferase Reporter Assay System (E1910, Promega) in a BioTek Synergy HT microplate reader.

6 Conclusion and Outlook

Chondrosarcoma is a malignant tumor that can arise from preexisting benign chondrogenic neoplasms with a dismal prognosis. The current and preferred protocol for chondrosarcoma treatment is wide surgical excision of tumors as neither chemo- nor radiotherapeutics regimes have shown a clinical benefit for patients during the last decades. Therefore, for those many cases where chondrosarcoma tumors recur, develop metastases or cannot be removed, the treatment options are extremely limited. On top of this concern, there is a big gap of knowledge regarding the genetic events that are required for chondrosarcoma tumors to develop and progress. Ergo, biomarkers for the early detection and accurate categorization of chondrogenic tumors are very much needed. This has a profound clinical impact, since it is extremely difficult for oncopathologists to accurately determine correct specific tumor subtypes (for example, enchondromas vs chondrosarcomas Grade 1), which would require different clinical interventions. Thus, there is an urgent need for the development of targeted therapies and reliable biomarkers, which can potentially be translated into a better clinical management of chondrosarcoma.

Consequently, the main interest of this thesis was set on identifying miRNAs (and the targets they regulate) that contribute to chondrosarcoma pathogenesis. As we wanted our research to be clinically –and potentially translationally- relevant, we did the initial identification of deregulated miRNAs from a cohort of tumors that included human chondrosarcoma specimens of different grades as well as benign chondrogenic tumors that are known to progress to chondrosarcoma (namely, enchondromas and osteochondromas).

The data from the studies carried out provide solid evidence for the aberrant downregulation of the miRNA cluster miR-143/145 in chondrosarcoma, whose levels

inversely correlates with tumor aggressiveness. Our studies demonstrate that miR-143/145 plays a key tumor suppressor role in chondrosarcoma by affecting various phenotypic features that contribute to cancer aggressiveness, namely adhesion to ECM substrates, anchorage-independent growth and cell cycle. This behavior was reflected in the tumor growth inhibition upon miR-143/145 upregulation in a paratibial mouse orthotopic xenograft model, which was previously established as a sub-aim of the present thesis. Importantly, the direct gene target of both cluster members, FSCN1, is being affected from the miR-143/145 deregulation by showing significantly increased expression levels in more advanced chondrosarcoma tumors. The role of FSCN1 has been poorly described in the field of bone sarcomas and ours is the first piece of data demonstrating its relevance in chondrosarcoma. Moreover, our data from loss-of-function studies provide strong evidence that FSCN1 contributes to chondrosarcoma malignancy, underlying the miR-143/145:FSCN1 axis as an attractive and novel target for the development of therapies. Of note, the functional link between FSCN1 and miR-143/145 is currently being tested in combinatorial experiments as part of the revision process of the manuscript (Results 5.2)

Future studies should consider the biological role of FSCN1 in *in vivo* settings in order to assess the potential role of this gene in primary tumor growth and/or metastatic disease in chondrosarcoma. In other epithelial cancer types, FSCN1 upregulation has been found to be clinically relevant (Yoder, Tso et al. 2005; Vignjevic, Schoumacher et al. 2007; Hashimoto, Kim et al. 2011). Importantly, *in vivo* studies have shown that FSCN1 gene-knockout mice are normal (Yamakita, Matsumura et al. 2009). Over the past years, few FSCN1 inhibitors have been developed and evaluated in breast tumor models (Chen, Yang et al. 2010; Han, Huang et al. 2016). These small molecules were shown to block acting binding/bundling activities of FSCN1 resulting in reduced tumor cell migration rates and metastatic spread *in*

vivo. Very importantly, one of these drugs (NP-G2-044) will be tested in a Phase I study to determine the safety of the drug when given orally in patient with advanced or metastatic solid tumors (NCT03199586, recruiting patients).

A substantial amount of the work presented in this thesis was focused on testing the potential use of circulating miRNAs as non-invasive biomarkers in chondrosarcoma. Our data provides strong proof that circulating miRNAs can be valuable tools for the improvement of the actual dismal diagnosis of chondrosarcoma and chondrogenic tumors. Our work demonstrates that miR-143/145 levels in plasma are tumor-malignancy dependent and, similar to what was observed in tumor samples, circulating miR-143/145 levels are lower in plasma of patients with more aggressive chondrosarcoma tumors. Moreover, circulating miR-145 was shown to be powerful for the distinction of benign versus malignant chondrogenic tumors. We believe that by combining multiple miRNAs into one signature, it could be possible to increase the sensitivity of our one-miRNA biomarker approach (miR-145) and lead to more accurate and earlier tumor detection and diagnosis, potentially leading to curative treatments and longer survival of chondrosarcoma patients. Additionally, aiming to increase the accuracy of tumor subtype diagnosis, circulating miRNA approaches can be combined with different imaging modalities in a multi-variable protocol, as it has been already implemented for other diseases (Nasser, Ranade et al. 2009; Regev, Healy et al. 2017).

We believe that future efforts focused on the elucidation of the mechanisms governing miR-143/145 regulation in chondrosarcoma can be a valuable step towards understanding at which stage of the disease the deregulation occurs and which factors control it. It has been previously shown in other cancer models that miR-143/145 expression is regulated transcriptionally and post-transcriptionally by multiple mechanisms (Zeinali, Mansoori et al. 2019). Furthermore, we believe that the global-miRNA signature generated by Next-

Generation sequencing in this thesis constitutes a valuable source for the identification of additional novel miRNA candidates that may contribute to chondrosarcoma progression. Our data also includes a list of upregulated miRNA candidates that, to this date, have been sparsely investigated in the field of chondrosarcoma and should be addressed in future studies. Interestingly, over-expressed miRNAs can be silenced *in vivo* by antagomirs - chemically engineered oligonucleotides that are complementary to miRNAs- opening up the possibility to test novel therapeutic approaches in chondrosarcoma (Krutzfeldt, Rajewsky et al. 2005).

Taken together, based on the results presented in this thesis it can be concluded that the miR-143/145 cluster serves as a potent tumor-suppressor in chondrosarcoma. Both miRNAs exert their antitumor functions, at least in part, through a coordinated regulation of FSCN1 that we subsequently recognized as a malignancy promoting factor in chondrosarcoma pathogenesis. This work suggests that FSCN1 inhibition or miR-143/145 restoration may hold potential as novel therapeutic approaches to chondrosarcoma. Moreover, we newly identified circulating miR-145 as a promising non-invasive biomarker, and its use in combination with other miRNAs or with chondrosarcoma histopathological or imaging features holds promise for the development of novel approaches with higher diagnostic accuracy.

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